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THE TARGETING OF CORTICOSTEROIDS
TO INFLAMED TISSUES

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Doctor of Philosophy

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SUMMARY

In recent years, much interest has focused on the beneficial effects of administering potentially harmful therapeutic agents in drug carriers so as to reduce their toxic side effects. Rheumatoid arthritis is a chronic systemic disease with progressive destruction of the joints and long-term patient disability. Corticosteroids have been shown to retard the progression of joint destruction but are limited in their use due to adverse side effects. This project, following the line of investigation started by other workers, was designed to study the use of microspheres to deliver corticosteroids to inflamed tissues by both the oral and intravenous routes. Hydrocortisone (HC)-loaded albumin microspheres were prepared by three different methods, by direct incorporation of HC within the particles, by indirect incorporation of HC by the enzymatic conversion of hydrocortisone-21-phosphate (H-21-P) to HC within the particles, and by the adsorption of HC onto the surface. HC was also loaded within PLA microspheres. The level of corticosteroid loading and *in vitro* release from microspheres was determined by HPLC analysis. A reversed-phase, ion-pairing HPLC method was developed to simultaneously measure both HC and H-21-P. The highest level of corticosteroid loading was achieved using the incorporation of H-21-P with enzymatic conversion to HC method. However, HPLC analysis showed only 5% of the incorporated steroid was HC. *In vitro* release rates of steroid from albumin microspheres showed >95% of incorporated steroid was released within 2 hours of dissolution. Increasing the protein:steroid ratio, and the temperature and duration of microsphere stabilization, had little effect on prolonging drug release. *In vivo* studies, using the carrageenan-induced rat hind-paw model of inflammation, indicated steroid-incorporated microspheres administered both orally and intraperitoneally were not therapeutically advantageous when compared to equivalent free steroid doses.

The ability of orally and intravenously dosed [¹²⁵I]-albumin microspheres (2.67 μm mean diameter) to accumulate in acutely and chronically inflamed tissues was investigated. The subcutaneous air-pouch was the model of inflammation used, with carrageenan as the inflammatory stimulus. Acute and chronic inflammation was shown to be consistently formed in pouch tissues in terms of cell infiltration and fluid exudate formation in the pouch cavity. Albumin microspheres were shown to accumulate in the inflamed tissues and pouch fluids after both oral and intravenous administration. Preliminary, confirmatory studies using latex microspheres and quantitation by GPC analysis, also indicated microsphere accumulation in both acutely and chronically inflamed air-pouch tissues. The results indicate the uptake and transfer of microspheres across the gastrointestinal tract into the circulation and their migration through disrupted endothelium and basement membranes at the inflamed sites.

KEY WORDS: Drug delivery; Rheumatoid arthritis; Microspheres; Hydrocortisone; Inflammation

In memory of

Dr David Arthur Lewis BSc MSc PhD DSc
1932 - 1994

To Mark

**Open a new window
Open a new door
Travel a new highway
That's never been travelled before...**

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Enterprise

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CONTENTS

	PAGE
Title	1
Thesis Summary	2
Dedication	3
Acknowledgements	5
Contents	6
List of Figures	14
List of Tables	20
List of Plates	24
Abbreviations	25
1. INTRODUCTION	27
1.1 Rheumatoid Arthritis	28
1.1.1 Predisposing Factors	28
1.1.2 Aetiologic Factors	28
1.1.3 Physiology Of The Knee Joint	29
1.1.4 The Early Immune Response In Rheumatoid Synovium	30
1.1.5 Early Disease Manifestations In The Rheumatoid Joint	31
1.1.6 Destruction Of Cartilage By Pannus In The Rheumatoid Joint	32
1.1.7 Clinical Features	33
1.1.8 Diagnosis Of Rheumatoid Arthritis	33
1.1.9 Animal Models Of Rheumatoid Arthritis	34
1.2 The Treatment Of Rheumatoid Arthritis	35
1.2.1 Non-Steroidal Anti-Inflammatory Drugs	35
1.2.2 Disease-Modifying Anti-Rheumatic Drugs	36
1.2.2.1 Gold Compounds	36
1.2.2.2 D-Penicillamine	37
1.2.2.3 Antimalarials	37
1.2.2.4 Methotrexate	38
1.2.2.5 Cyclosporin A	38
1.2.3 Cytotoxic Therapy	38
1.2.4 Corticosteroid Therapy	38
1.2.4.1 Clinical Pharmacology And Pharmacokinetics	39
1.2.4.2 Mode Of Action	41
1.2.5 Surgical Treatment	43
1.2.6 Immunotherapy	44

1.2.7	Laser Therapy	44
1.2.8	Dietary Therapy	44
1.3	The Need For Greater Site-Specificity	45
1.3.1	Local Administration	46
1.3.2	Site-Specific Drug Delivery	46
1.4	Particulate Drug Carrier Systems	47
1.4.1	Albumin Microspheres	48
1.4.2	Polylactic Acid Microspheres	51
1.5	Anatomical Barriers To Particulate Drug Delivery	54
1.5.1	The Endothelial Barrier	54
1.5.2	The Mononuclear Phagocyte System	55
1.6	Microsphere Uptake After Intravenous Administration	56
1.6.1	Strategies For Avoiding The MPS	57
	1.6.1.1 MPS Suppression	57
	1.6.1.2 Alteration Of Particle Surface Characteristics	58
1.7	Microsphere Uptake After Oral Administration	59
1.7.1	The Transcellular Route	60
1.7.2	The Paracellular Route	61
1.7.3	Uptake By M-Cells And The Peyer's Patches	62
1.7.4	Translocation Into The Bloodstream	64
1.8	Origins And Scope Of Work	64
2.	PREPARATION AND CHARACTERIZATION OF ALBUMIN AND POLYLACTIC ACID MICROSPHERES	66
2.1	Introduction	67
2.2	Materials	68
2.3	Instrumentation	68
2.4	Methods	69
2.4.1	Albumin Microsphere Preparation	69
	2.4.1.1 Thermal-Denatured Albumin Microsphere Preparation	69
	2.4.1.2 Chemically Cross-Linked Albumin Microsphere Preparation	71
	2.4.1.3 Radiolabelled Albumin Microsphere Preparation	72
2.4.2	PLA Microsphere Preparation	72
2.4.3	Particle Size Analysis	74
2.4.4	Adsorption Of Poloxamine 908 Onto Albumin Microspheres	74
2.4.5	Zeta Potential Measurement Of Albumin Microspheres	75

2.5	Results And Discussion	77
2.5.1	Albumin Microsphere Preparation	77
2.5.2	PLA Microsphere Preparation	83
2.5.3	Adsorption Of Poloxamine 908 Onto Chemically Cross-Linked Albumin Microspheres	84
2.6	Summary	87
3.	DEVELOPMENT OF A HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE SIMULTANEOUS, QUANTITATIVE ANALYSIS OF HYDROCORTISONE AND HYDROCORTISONE-21-PHOSPHATE	88
3.1	An Introduction To High-Performance Liquid Chromatography	89
3.1.1	Mathematical Parameters	92
3.2	Materials	94
3.3	Instrumentation	94
3.3.1	Analytical	94
3.3.2	Subsidiary	94
3.4	Experimental	95
3.4.1	Determination Of Optimum UV Wavelength For Analyte Detection	95
3.4.2	Determination Of Mobile Phase Composition For Separation Of Hydrocortisone And Hydrocortisone-21-Phosphate	96
3.4.3	Study Of Internal Standard Candidates	97
3.4.4	Quantitative Analysis Of Hydrocortisone and Hydrocortisone-21-Phosphate	100
3.5	Summary	103
4.	STEROID-INCORPORATED ALBUMIN AND POLYLACTIC ACID MICROSPHERES	104
4.1	Introduction	105
4.2	Materials	106
4.3	Instrumentation	106
4.3.1	Analytical	106
4.3.2	Subsidiary	106
4.4	Methods	107
4.4.1	Direct Incorporation Of Hydrocortisone In Albumin Microspheres	107

4.4.2	Indirect Incorporation Of Hydrocortisone By Enzymatic Cleavage Of Hydrocortisone-21-Phosphate In Albumin Microspheres	107
4.4.3	Adsorption Of Hydrocortisone Onto Empty Albumin Microspheres	108
4.4.4	Incorporation Of Hydrocortisone In PLA Microspheres	108
4.4.5	Extraction And Quantitation Of Steroid Incorporated In Albumin Microspheres	109
4.4.5.1	Methanol Reflux	109
4.4.5.2	Trypsin Degradation	109
4.4.6	Extraction And Quantitation Of Steroid Incorporated In PLA Microspheres	110
4.4.7	Determination Of Steroid Release Profiles From Albumin And PLA Microspheres	110
4.4.8	Determination Of Hydrocortisone Solubility In Various Solvent Systems	110
4.4.9	Particle Size Analysis	111
4.4.10	Examination Of Steroid-Incorporated Albumin Microspheres By Transmission Electron Microscopy	111
4.5	Experimental	111
4.5.1	Effect Of Steroid:Albumin Ratio On Steroid-Incorporation Levels And Steroid Release Profiles From Thermal-Denatured Albumin Microspheres	111
4.5.2	Effect Of Denaturation Time On Steroid-Incorporation Levels And Steroid Release Profiles From Thermal-Denatured Albumin Microspheres	112
4.5.3	Effect Of Denaturation Temperature On Steroid-Incorporation Levels And Steroid Release Profiles From Thermal-Denatured Albumin Microspheres	113
4.5.4	Effect Of Steroid:Polymer Ratio On Steroid-Incorporation Levels In PLA Microspheres	113
4.5.5	Effect Of Steroid Saturation Of The Aqueous Phase On Steroid-Incorporation Levels In PLA Microspheres	113
4.5.6	Effect Of Increasing Steroid Solubility In Various Solvents On Steroid-Incorporation Levels In PLA Microspheres	114
4.5.7	Drug Release Profiles From Steroid-Incorporated PLA Microspheres	114
4.6	Results And Discussion	115

4.6.1	Steroid-Incorporation In Thermal-Denatured Albumin Microspheres	115
4.6.1.1	Effect Of Steroid:Albumin Ratio On Steroid- Incorporation Levels And Steroid Release From Thermal-Denatured Albumin Microspheres	119
4.6.1.2	Effect Of Thermal-Denaturation Time On Steroid- Incorporation Levels And Steroid Release From Albumin Microspheres	121
4.6.1.3	Effect Of Denaturation Temperature On Steroid- Incorporation Levels And Steroid Release From Thermal-Denatured Albumin Microspheres	123
4.6.2	Hydrocortisone-Incorporation In PLA Microspheres	124
4.6.2.1	Effect Of Hydrocortisone:Polymer Ratio On Hydrocortisone-Incorporation Levels In PLA Microspheres	124
4.6.2.2	Use Of A Co-Solvent System To Increase Hydrocortisone Incorporation Levels In PLA Microspheres	124
4.6.3	Steroid Release Profiles From PLA Microspheres	127
4.7	Summary	128
5.	TISSUE DISTRIBUTION OF ALBUMIN MICROSPHERES FOLLOWING ORAL AND INTRAVENOUS ADMINISTRATION TO RATS INDUCED WITH AN EXPERIMENTAL MODEL OF INFLAMMATION	130
5.1	Introduction To The Targeting Of Microspheres To Sites Of Inflammation	131
5.1.1	The Subcutaneous Air-Pouch Model	133
5.1.2	Principle Uses Of The Subcutaneous Air-Pouch Model	134
5.2	Materials	135
5.2.1	Animals	135
5.2.2	Subsidiary	135
5.3	Methods	135
5.3.1	Anaesthesia	135
5.3.2	Formation Of 6-Day Old Subcutaneous Air-Pouches	136
5.3.2.1	Formation Of Acute Inflammation In 6-Day Old Subcutaneous Air-Pouches	136
5.3.2.2	Formation Of Chronic Inflammation In 6-Day Old Subcutaneous Air-Pouches	136

5.3.3	Preparation Of Radiolabelled Albumin Microspheres	137
5.3.4	Preparation Of Surface Modified Radiolabelled Albumin Microspheres	137
5.4	Experimental	137
5.4.1	Characterization Of Acute And Chronic Inflammation Induced In Subcutaneous Air-Pouches	137
5.4.1.1	Study Of Acute Inflammation In The Rat Subcutaneous Air-Pouch Model	138
5.4.1.2	Study Of Chronic Inflammation In The Rat Subcutaneous Air-Pouch Model	138
5.4.2	Determination Of Increased Vascular Permeability And Blood Vessel Formation In Subcutaneous Air-Pouch Tissues With Acute And Chronic Inflammation	139
5.4.2.1	Study Of Increased Vascular Permeability Using Radioiodinated Serum Albumin As A Marker Of Vascular Permeability	139
5.4.2.2	Study Of Increased Blood Vessel Formation In Air-Pouch Tissues By Direct Examination	140
5.4.3	Determination Of Albumin Microsphere Distribution To Subcutaneous Air-Pouch Tissues With Acute And Chronic Inflammation Following Oral And Intravenous Administration	140
5.4.3.1	Intravenous Administration	141
5.4.3.2	Oral Administration	141
5.5	Results And Discussion	142
5.5.1	Characterization Of Acute And Chronic Inflammation Induced In Subcutaneous Air-Pouch Tissues	142
5.5.2	Determination Of Increased Vascular Permeability And Blood Vessel Formation In Subcutaneous Air-Pouch Tissues	145
5.5.3	Tissue Distribution Of Orally And Intravenously Administered Albumin Microspheres	149
5.5.3.1	Tissue Distribution Of Intravenously Administered Microspheres	149
5.5.3.2	Tissue Distribution Of Orally Administered Microspheres	153
5.6	Summary	158

6.	TISSUE DISTRIBUTION OF LATEX MICROSPHERES AFTER ORAL AND INTRAVENOUS ADMINISTRATION TO RATS INDUCED WITH AN EXPERIMENTAL MODEL OF INFLAMMATION	161
6.1	Introduction	162
6.1.1	Gel Permeation Chromatography	162
6.2	Materials	164
6.2.1	Latex Microspheres	164
6.2.2	Animals	164
6.3	Instrumentation	164
6.3.1	Analytical	164
6.3.2	Subsidiary	165
6.4	Methods	165
6.4.1	Anaesthesia	165
6.4.2	Formation Of 6-Day Old Subcutaneous Air-Pouches	165
6.4.3	Polystyrene Extraction Process From Tissue Samples	165
6.4.4	Calibration And Sensitivity Studies	166
6.5	Experimental	167
6.5.1	Tissue Distribution Of Orally Dosed Latex Microspheres	167
6.5.2	Tissue Distribution Of Intravenously Administered Latex Microspheres	167
6.6	Results And Discussion	168
6.6.1	Calibration And Sensitivity Studies	168
6.6.2	Tissue Distribution Of Orally Dosed And Intravenously Administered Latex Microspheres	172
6.7	Summary	175
7.	USE OF THE CARRAGEENAN-INDUCED RAT HIND-PAW MODEL OF INFLAMMATION TO DETERMINE THE THERAPEUTIC USE OF STEROID-INCORPORATED ALBUMIN MICROSPHERES	176
7.1	Introduction	177
7.1.1	Carrageenan-Induced Oedema In The Rat Hind-Paw For Testing The Efficacy Of Anti-Inflammatory Drugs	177
7.2	Materials	178
7.2.1	Albumin Microspheres	178
7.2.2	Steroid-Incorporated Albumin Microspheres	178
7.2.3	Animals	178
7.3	Instrumentation	178
	7.3.1 Analytical	178

	7.3.2	Subsidiary	179
7.4		Methods	179
	7.4.1	Anaesthesia	179
	7.4.2	Carrageenan-Induced Hind-Paw Inflammation In The Rat	179
	7.4.3	Measurement Of Oedema Formation In The Rat Hind-Paw Model Of Inflammation	179
7.5		Experimental	180
	7.5.1	Study Of The Ability Of Orally Dosed Steroid-Incorporated Albumin Microspheres To Reduce Oedema Formation In The Carrageenan-Induced Rat Hind-Paw Model Of Inflammation	180
	7.5.2	Study Of The Ability Of Intra-Peritoneally Administered Steroid-Incorporated Albumin Microspheres To Reduce Oedema Formation In The Carrageenan-Induced Rat Hind-Paw Model Of Inflammation	181
7.6		Results And Discussion	182
	7.6.1	Determination Of The Ability Of Orally Dosed Steroid-Incorporated Albumin Microspheres To Reduce Oedema Formation In Carrageenan-Induced The Rat Hind-Paw Model Of Inflammation	182
	7.6.2	Determination Of The Ability Of Intra-Peritoneally Administered Steroid-Incorporated Albumin Microspheres To Reduce Oedema Formation In The Carrageenan-Induced Rat Hind-Paw Model Of Inflammation	183
7.7		Summary	185
8.		CONCLUDING REMARKS	187
9.		REFERENCES	192
10.		APPENDICES	212

LIST OF FIGURES

		PAGE
Figure 1.1	Schematic Diagram Of The Synovial Joint (from Barnett <i>et al.</i> , 1961)	29
Figure 1.2	Proposed Mechanism Of Glucocorticoid Inhibition Of Pro-Inflammatory Mediators (from George & Kirwan, 1990)	42
Figure 1.3	Schematic Representation Of The Steps Followed During The Manufacture Of Thermal-Denatured And Chemically Cross-Linked Albumin Microspheres (adapted from the methods reported by Gallo <i>et al.</i> , 1984; Yapel, 1985)	50
Figure 1.4	Synthesis Of DL-Lactide Monomers (a) And Ring Opening Polymerization Of DL-Lactide (b) (adapted from Wise <i>et al.</i> , 1979)	52
Figure 1.5	Microsphere Formation By The Emulsification/Solvent Evaporation Technique (adapted from Watts <i>et al.</i> , 1990)	53
Figure 1.6	Schematic Diagram Of The Endothelial Cell Barrier At Different Sites (adapted from Wright & Illum, 1992)	55
Figure 1.7	Schematic Representation Of The Proposed Mechanisms Of Particulate Uptake Across The Gastrointestinal Tract. (1) The Transcellular Route, (2) The Paracellular Route, (3) Transport <i>Via</i> The M-Cells Overlying The Peyer's Patches (adapted from Florence & Jani, 1993)	61
Figure 1.8	Schematic Representation Of The Structure Of Intestinal Peyer's Patches Showing M-Cell Location In The Follicle-Associated Epithelium (adapted from O'Hagan, 1990)	63
Figure 2.1	Particle Size Distribution Of Thermal-Denatured Albumin Microspheres Prepared By Method C. Geometric Mean Diameter = 2.47 μm ; σ_g = 1.84 μm	79

Figure 2.2	Particle Size Distribution Of Chemically Cross-Linked Albumin Microspheres Prepared By Method G. Geometric Mean Diameter = 2.02 μm ; σ_g = 0.62 μm	82
Figure 2.3	Particle Size Distribution Of PLA Microspheres Prepared By Method I. Geometric Mean Diameter = 2.53 μm ; σ_g = 1.59 μm	85
Figure 2.4	Surface Charge Of Various Formulations Of Chemically Cross-Linked Albumin Microspheres Coated With Poloxamine 908 Measured As Zeta Potential (mean \pm sd; n = 3) A) AZ55 Standard Latex Particles In AZ55 Buffer, B) Polyscience Latex Microspheres, C) Glutaraldehyde Cross-Linked Albumin Microspheres, D) Glutaraldehyde Cross-Linked Albumin Microspheres Coated With Poloxamine 908 (2.0% w/v), E) Glutaraldehyde Cross-Linked Albumin Microspheres Coated With Poloxamine 908 (0.2% w/v). B-D 37 mM Phosphate Buffered Saline, pH 7.2	86
Figure 2.5	The Structure Of Poloxamine 908 And A Model For Their Adsorption To Particles (adapted from Wright & Illum, 1992) PEO - Polyoxyethylene, PPO - Polyoxypropylene *Molecular Blocks (Average value in moles) PEO-PPO 122-22	87
Figure 3.1	The Chemical Structures Of Hydrocortisone And Hydrocortisone-21-Phosphate Disodium Salt (adapted from Florey, 1983)	89
Figure 3.2	The Basic Components Of A High-Performance Liquid Chromatography System (adapted from Li Wan Po & Irwin, 1980)	90
Figure 3.3	A Typical Chromatogram For A Two Component Separation Where t_A And t_B Are The Retention Times Of Components A And B Respectively, And t_0 Is The Retention Time Of Any Unretained Solute (adapted from Li Wan Po & Irwin, 1980)	91

Figure 3.4	UV Absorbance Profiles Of Hydrocortisone, Hydrocortisone-21-Phosphate And Internal Standard Candidates In Methanol Between 190-350 nm	95
Figure 3.5	The Effect On Hydrocortisone And Hydrocortisone-21-Phosphate Chromatographic Separation By Increasing The Percentage Of Methanol In The Mobile Phase From 45-65% v/v In Water	98
Figure 3.6	A Typical Chromatogram For The Separation Of Hydrocortisone And Hydrocortisone-21-Phosphate With Propyl Paraben As An Internal Standard	101
Figure 3.7	A Typical Calibration Plot For Hydrocortisone (\blacktriangle) And Hydrocortisone-21-Phosphate (Δ) Quantitative Analysis By HPLC Using Propyl Paraben As An Internal Standard	102
Figure 4.1	A Hypothetical Picture Of An Albumin Microsphere Showing Possible Sites Of Location Of Drug (adapted from Gupta <i>et al.</i> , 1986a)	115
Figure 4.2	Release Of Steroid From Thermal-Denatured ($125\pm 5^\circ\text{C}$, 30 min) Albumin Microspheres Prepared With Varying Steroid:Albumin Ratios. Dissolution In 0.0067M Phosphate Buffer, pH 7.0, 37°C , Under Sink Conditions. Each point represents mean ($n=3$). Error bars representing standard deviation from the mean have been omitted for clarity	121
Figure 4.3	Release Of Steroid From Thermal-Denatured Albumin Microspheres Prepared With Varying Time Lengths Of Thermal Denaturation ($125\pm 5^\circ\text{C}$) Using A Steroid:Albumin Ratio Of 1:1.25. Dissolution In 0.0067M Phosphate Buffer, pH 7.0, 37°C , Under Sink Conditions. Each point represents mean ($n=3$). Error bars representing standard deviation from the mean have been omitted for clarity	122

- Figure 4.4 Release Of Steroid From Thermal-Denatured (1.5 hr) Albumin Microspheres Prepared With Varying Temperatures Of Thermal Denaturation. Dissolution In 0.0067M Phosphate Buffer, pH 7.0, 37°C, Under Sink Conditions. Each point represents mean (n=3). Error bars representing standard deviation from the mean have been omitted for clarity 124
- Figure 4.5 Release Of Steroid From PLA Microspheres. Dissolution In 0.0067M Phosphate Buffer, pH 7.0, 37°C, Under Sink Conditions. Each point represents mean (n=3). Error bars representing standard deviation from the mean have been omitted for clarity. 1 - No saturation of the external phase, 2 - HC Saturation of the external phase, 3 - 5% DMSO in the internal phase, no saturation of the external phase, 4 - 5% DMSO in the internal phase, HC saturation of the external phase. 127
- Figure 5.1 Pouch Fluid Exudate Volumes (▲) Collected Over A 10 Day Period From Rat Subcutaneous Air-Pouches Injected With 2 ml 2% w/v Sterile Carrageenan Solution. Each value represents the mean \pm sd (n=3 rats) 143
- Figure 5.2 Total Cell Numbers (▲) And % PMNL (Δ) In Pouch Fluid Exudates Collected From Rat Subcutaneous Air-Pouches 2, 4, 6, 8, And 24 Hours After The Injection of 2 ml 2% w/v Sterile Carrageenan Solution. Each value represents the mean \pm sd (n=3 rats) 144
- Figure 5.3 Total Cell Numbers (▲) And % PMNL (Δ) In Pouch Fluid Exudates Collected From Rat Subcutaneous Air-Pouches 1, 3, 7, And 10 Days After The Injection of 2 ml 2% w/v Sterile Carrageenan Solution. Each value represents the mean \pm sd (n=3 rats) 144

- Figure 5.4 Tissue Distribution Of [125 I]-Albumin Microspheres (mean diameter 2.30-2.75 μ m) 4 Hours After Intravenous Administration *Via* Tail Vein Injection. Graphs A And C - Microspheres With No Coating. Graphs B And D - Microspheres Coated With Poloxamine 908. Each bar represents the mean \pm sd (n=3). ¹Significantly different from control tissue. ²Significantly different from plain microspheres. ³Significantly different from Poloxamine 908-coated microspheres. *P<0.005 **P<0.01 ***P<0.05 150
- Figure 5.5 Tissue Distribution Of [125 I]-Albumin Microspheres (Geometric Mean Diameter And σ g 2.67 \pm 1.28 μ m) 4 Hours After Oral Administration By Gavage Needle. A And C - Microspheres With No Coating. B And D - Microspheres Coated With Poloxamine 908. Each bar represents the mean \pm sd (n=3 rats). ¹Significantly different from control tissue. **P<0.01 154
- Figure 5.6 Tissue Distribution Of [125 I]-Albumin Microspheres (Geometric Mean Diameter And σ g 2.67 \pm 1.28 μ m) 24 Hours After Oral Administration By Gavage Needle. A And C - Microspheres With No Coating. B And D - Microspheres Coated With Poloxamine 908. Each bar represents the mean \pm sd (n=3 rats). ¹Significantly different from control tissue. ²Significantly different from acute fluid. ³Significantly different from plain microspheres. *P<0.1 **P<0.05 ***P<0.005 156
- Figure 6.1 Plot To Show The Relationship Between The Molecular Weight And Retention Time (min) Of A Standard Mixture Of Polystyrene (EasiCal[®], Polymer Laboratories Ltd., Shropshire, UK) With A Range Of Molecular Weights, Analysed By Gel Permeation Chromatography Each point represents mean \pm sem (n=4). Regression correlation coefficient = 0.999 169

- Figure 6.2 Typical Calibration Plots For Quantitative Analysis Of
Latex Microspheres In THF (\blacktriangle), Blood (Δ), And Liver (\square)
By Gel Permeation Chromatography
Each point represents mean \pm sd (n=3) 170
- Figure 6.3 Typical Gel Permeation Chromatogram For Polystyrene Extracted
From A Liver Tissue Sample 171

LIST OF TABLES

		PAGE
Table 1.1	Source Of Some Chemoattractants Produced And Released In The Rheumatoid Joint (from Harris, 1988)	31
Table 1.2	Actions Of Leukotrienes And Prostaglandins Produced By Activated PMNL In Rheumatoid Synovium	32
Table 1.3	1987 Criteria For Diagnosis of Rheumatoid Arthritis (from Arnett <i>et al.</i> , 1988)	34
Table 1.4	Comparison Of Commonly Used Glucocorticoids In RA Therapy (from George & Kirwan, 1990)	40
Table 1.5	Some Pharmacological Actions Of Glucocorticoids (adapted from George & Kirwan, 1990)	41
Table 1.6	Pharmacological Effects Of Glucocorticoids On The Cellular Components Of The Immune System	43
Table 1.7	Some Examples Of Microsphere/Nanosphere Drug Delivery Systems Described In The Literature (adapted from Davis & Illum, 1989)	44
Table 1.8	Proposed Mechanisms Of Uptake Of Particulates Across The Gastrointestinal Tract (adapted from O'Hagan, 1990)	60
Table 2.1	Modifications To Thermal-Denatured Albumin Microsphere Preparation Methods Cited In The Literature	70
Table 2.2	Modifications To Chemically Cross-Linked Albumin Microsphere Preparation Methods Cited In The Literature	71
Table 2.3	Modifications To Some PLA Microsphere Preparation Methods Reported In The Literature Based On The Original Solvent Evaporation Method Of Beck <i>et al.</i> , (1979)	73

Table 2.4	Parameters Used In Zeta Potential Measurements	74
Table 2.5	Factors Affecting Size And Size Distribution Of Albumin Microspheres (from Gupta & Hung, 1989a)	78
Table 2.6	Mean Particle Size Obtained With Some Variations Of The Methods Cited In The Literature Used To Prepare Thermal-Denatured Albumin Microspheres	80
Table 2.7	Mean Particle Size Obtained With Some Variations Of The Methods Cited In The Literature Used To Prepare Glutaraldehyde Cross-Linked Albumin Microspheres	81
Table 2.8	Mean Particle Size Obtained With Some Variations Of The Methods Cited In The Literature Used To Prepare PLA Microspheres	84
Table 3.1	Maximum UV Absorbance Wavelengths And Extinction Coefficients At 242 nm For Hydrocortisone, Hydrocortisone-21-Phosphate And Candidate Internal Standard Compounds In Methanol	96
Table 3.2	The Effect Of Increasing The Percentage Of Methanol In The Mobile Phase On The Simultaneous Analysis Of Hydrocortisone And Hydrocortisone-21-Phosphate By HPLC In Terms Of Retention Time, Chromatographic Efficiency And Compound Resolution	99
Table 3.3	Retention Times Of Internal Standard Candidate Compounds Analysed Simultaneously With Hydrocortisone And Hydrocortisone-21-Phosphate By HPLC	100
Table 3.4	Corresponding Calibration Statistics For Figure 3.7	102
Table 4.1	Comparison Of Three Methods Of Hydrocortisone-Incorporation In Thermal-Denatured ($125\pm 5^{\circ}\text{C}$, 30 min) Albumin Microspheres Each figure represents the mean \pm sd (n=3)	118

Table 4.2	Effect Of Steroid:Albumin Ratio On Steroid-Incorporation Levels And Particle Size Of Thermal-Denatured ($125\pm 5^{\circ}\text{C}$, 30 min) Albumin Microspheres Each figure represents the mean \pm sd (n=3)	120
Table 4.3	Effect Of Increasing Thermal-Denaturation ($125\pm 5^{\circ}\text{C}$) Time On Steroid-Incorporation And Particle Size In Albumin Microspheres Each figure represents the mean \pm sd (n=3)	122
Table 4.4	Effect Of Increasing Thermal-Denaturation (1.5 hr) Temperature On Steroid-Incorporation And Particle Size In Albumin Microspheres Each figure represents the mean \pm sd (n=3)	123
Table 4.5	Effect Of Hydrocortisone:Polymer Ratio On Hydrocortisone-Incorporation And Particle Size In PLA Microspheres Each figure represents the mean \pm sd (n=3)	125
Table 4.6	Determination Of Hydrocortisone Solubility At 25°C In Various Aqueous And Organic Solvent Systems	126
Table 4.7	The Effect Of Hydrocortisone Saturation Of The External Phase And The Use Of A Co-Solvent System In The Internal Phase On Hydrocortisone Incorporation In PLA Microspheres Each figure represents the mean \pm sd (n=3)	126
Table 5.1	The Percentage Of Dosed Radioactivity Per Gram Of Tissue In: (1) Control Air-Bleb Tissue, (2) Air-Pouch Tissue With No Irritant Administered, (3) Air-Pouch Tissue 24 Hours After The Injection Of 2 ml 2% w/v Sterile Carrageenan Solution (Acute Inflammation), And (4) Air-Pouch Tissue 72 Hours After The Injection Of 2 ml 2% w/v Sterile Carrageenan Solution (Chronic Inflammation). Each value represents the mean \pm sd (n=3 rats). ¹ Significantly different from air-bleb tissue. * $P\leq 0.005$,	146
Table 6.1	Calibration Statistics For Figure 6.2	170

- Table 6.2 The Percentage Of The Dose Of 220 nm Latex Microspheres Distributed To Various Organs Of The Body 24 hr After Oral Dosing In The Rat Induced With An Experimental Model Of Inflammation
For each study n=1 rat. Results are expressed as % of administered dose recovered from the whole organ unless otherwise stated 173
- Table 6.3 The Percentage Of The Dose Of 220 nm Latex Microspheres Distributed To Various Organs Of The Body 4 hr After I.V. Administration In The Rat Induced With An Experimental Model Of Inflammation
For each study n=1 rat. Results are expressed as % of administered dose recovered from the whole organ unless otherwise stated 174
- Table 7.1 The Percentage Increase In Hind-Paw Volume And The Percentage Reduction Of Oedema Formation In The Carrageenan-Induced Rat Hind-Paw By Orally Administered, Steroid-Incorporated, Albumin Microspheres (17.5 mg kg⁻¹) Measured 5 Hours After Irritant Injection Each figure represents mean ± sd (n=3 rats) ¹Significantly different from control plain albumin microspheres *P<0.005 182
- Table 7.2 The Percentage Increase In Hind-Paw Volume And The Percentage Reduction Of Oedema Formation In The Carrageenan-Induced Rat Hind-Paw By Intra-Peritoneally Administered, Steroid-Incorporated, Albumin Microspheres (17.5 mg kg⁻¹) Measured 5 Hours After Irritant Injection
Each figure represents mean ± sd (n=3 rats) ¹Significantly different from control plain albumin microspheres *P<0.005 **P<0.01 183

LIST OF PLATES

		PAGE
Plate 2.1	Scanning Electron Micrograph Of Thermal-Denatured Albumin Microspheres	79
Plate 2.2	Scanning Electron Micrograph Of Chemically Cross-Linked Albumin Microspheres	82
Plate 2.3	Scanning Electron Micrograph Of PLA Microspheres	85
Plate 4.1	Transmission Electron Micrograph Of Steroid-Incorporated Albumin Microspheres Prepared By Thermal-Denaturation At $125\pm 5^{\circ}\text{C}$ For 30 Minutes	117
Plate 4.2	Transmission Electron Micrograph Of Steroid-Incorporated Albumin Microspheres Prepared By Thermal-Denaturation At $125\pm 5^{\circ}\text{C}$ For 30 Minutes And Subjected To 30 Minutes Dissolution At 37°C	117
Plate 5.1	Blood Vessel Formation In Subcutaneous Air-Bleb Tissue	147
Plate 5.2	Blood Vessel Formation In Subcutaneous Air-Pouch Tissue	147
Plate 5.3	Blood Vessel Formation In Subcutaneous Air-Pouch Tissue 24 Hours After Injection Of 2 ml 2% w/v Sterile Carrageenan Solution	148
Plate 5.4	Blood Vessel Formation In Air-Pouch Tissue 72 Hours After Injection Of 2 ml 2% w/v Sterile Carrageenan Solution	148
Plate 5.5	Scanning Electron Micrograph Showing Albumin Microspheres Recovered From A Rat Urine Sample 24 Hours After Oral Administration Of Albumin Microspheres (Geometric Mean Diameter And σ_g $2.67\pm 1.28 \mu\text{m}$)	158

LIST OF ABBREVIATIONS

ARA	American Rheumatism Association
AUFS	Absorbance units full scale
BSA	Bovine serum albumin
CL	Column length
DMARDs	Disease modifying anti-rheumatic drugs
FAE	Follicle-associated epithelium
FITC	Fluorescein isothiocyanate
GIT	Gastrointestinal tract
GPC	Gel permeation chromatography
HC	Hydrocortisone
HE	Haematoxylin and Eosin stain
Hg	Mercury
HLA	Human leukocyte antigen
HPLC	High-performance liquid chromatography
H-21-P	Hydrocortisone-21-phosphate disodium salt
I	Iodine
i.a.	intra-articular
ICAM	Intercellular adhesion molecule
ID	Internal diameter
IFN- γ	Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin
i.m.	intramuscular
i.v.	intravenous
LT	Leukotriene
M	Molar
MBq	Megabecquerels
M-cell	Membranous epithelial cell
MeOH	Methanol
MHC	Major histocompatibility complex
MLN	Mesenteric lymph node
Mn	Number-average molecular weight
MNL	Mononuclear lymphocytes
MP	Mobile phase
MPS	Mononuclear phagocyte system
Mw	Weight-average molecular weight

MWD	Molecular weight distribution
Mwt	Molecular weight
Na	Sodium
NSAIDs	Non-steroidal anti-inflammatory drugs
ODS	Octadecylsilane
P	Probability
PBCA	Polybutylcyanoacrylate
PBS	Phosphate buffered saline
PG	Prostaglandin
PLA	Poly(lactic acid)
PLGA	Poly(lactic-co-glycolic acid)
PMNL	Polymorphonuclear leukocyte
PP	Peyer's patches
psi	pounds per square inch
PVA	Polyvinylalcohol
RA	Rheumatoid arthritis
RES	Reticuloendothelial system
RF	Rheumatoid factor
RNA	Ribonucleic acid
RP	Reversed-phase
rpm	revolutions per minute
s.c.	subcutaneous
sd	standard deviation
sem	standard error of the mean
SEM	Scanning electron microscopy
TCR	T-cell receptor
TCV	T-cell vaccination
TEM	Transmission electron microscopy
THF	Tetrahydrofuran
TNF- α	Tumour necrosis factor-alpha
UV	Ultra-violet
v/v	volume per volume
W	Watts
w/o	water-in-oil
w/v	weight per volume
w/w	weight per weight
$^{\circ}\text{C}$	degrees Celcius

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

1.1 RHEUMATOID ARTHRITIS

Rheumatoid arthritis (RA) is a chronic systemic disorder that is dominated by its serious and debilitating sequelae derived from the erosion of articular joint tissues. The disease commonly first attacks the small limb joints of the hands and feet, and may continue to affect the wrist, ankle, knee, shoulder, elbow and hip joints. RA has been reported in every part of the world with no geographical limits and no preference for climatic conditions. Its prevalence is considered to be approximately 1%, with population surveys showing an increasing incidence of RA in those between the ages of 40 and 60 years. RA occurs with an annual incidence of 3 in every 10,000 adult population (Spector, 1990), with about 10% of these patients becoming seriously disabled and 40% having significant disability.

1.1.1 PREDISPOSING FACTORS

The role of sex appears to be stronger than any geographical or racial factors as a determinant of the disease. Epidemiological and immunological evidence has suggested that hormonal factors may play a role in the course of RA with surveys showing an increased female-to-male predominance. Further evidence for a hormonal effect comes from the observation of an amelioration in the disease during pregnancy, first noted by Hench, (1938). The possible mechanisms thought to be involved in this phenomenon have recently been reviewed by Da Silva & Spector, (1992).

The propensity to develop RA also appears to be associated with class II antigens of the major histocompatibility complex (MHC). Genetic epidemiology studies have shown that in caucasoids, seropositive RA is associated with the HLA-DR4 gene locus on chromosome six, whilst in Jewish and Asian Indian populations the association is with the HLA-DR1 gene locus (Cooke & Scudamore, 1989; Silman, 1992).

1.1.2 ETIOLOGIC FACTORS

For a long time a virus or bacteria has been suspected of triggering the disease process in RA. Numerous pathogens have been implicated, recent attention concentrating on endogenous retroviruses (Krieg *et al.*, 1992) and intestinal bacteria (Hazenber *et al.*, 1992). If bacteria play a role in triggering and perpetuating RA, two possible mechanisms for this have been proposed: 1) an autoimmune reaction occurs against

bacterial antigens which show a high degree of homology with mammalian proteins (heat-shock proteins) (Lydyard *et al.*, 1990), or 2) an immune reaction against bacterial antigens present in the synovial tissue may occur (Hazenber *et al.*, 1992).

1.1.3 PHYSIOLOGY OF THE KNEE JOINT

Figure 1.1 shows diagrammatically the structure of a human synovial joint in the non-diseased state (Barnett *et al.*, 1961). The blood supply of the joint comes from large vessels traversing the fibrous joint capsule. Under this capsule lies a thin lining membrane constructed of a delicate arrangement of fine collagen fibres, capillaries and connective tissue cells. This synovial membrane lies on a cushion of fat cells termed the subsynovial layer. The inner synovial membrane, the intima, is predominantly cellular consisting of synoviocytes, fibroblasts, histiocytes and mast cells (Castor, 1960). It is changes in the histology of the joint that gives rise to the pathogenic processes occurring in rheumatoid synovium.

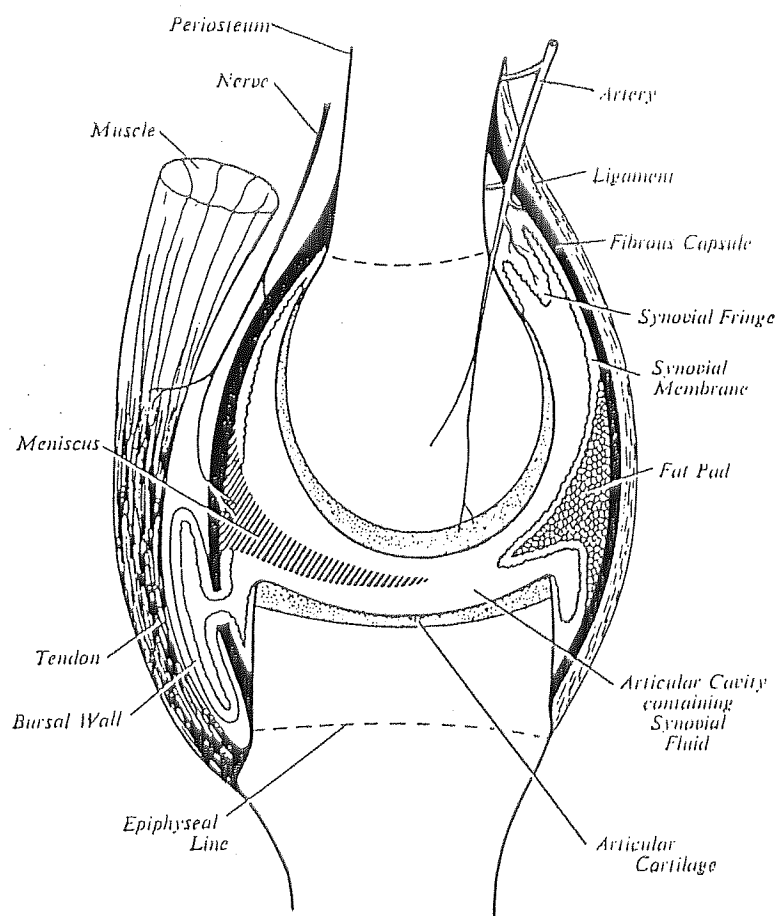


FIGURE 1.1 Schematic Diagram Of The Synovial Joint (from Barnett *et al.*, 1961)

1.1.4 THE EARLY IMMUNE RESPONSE IN RHEUMATOID SYNOVIUM

It seems probable that the antigen which triggers the disease must somehow migrate to, and localize in, the synovium. Synovial lining cells have been shown to be macrophage-like, with most expressing HLA-DR antigens, and are able to present antigen to T-lymphocytes resulting in their activation (Andriopoulos *et al.*, 1976). Lymphocytes are recruited to the synovium from the peripheral lymph nodes and traverse the synovial endothelial lining facilitated by the expression of specific membrane molecules on the mature T-lymphocytes (Rasmussen *et al.*, 1985) and on the endothelial cells (Wilder *et al.*, 1991). The activated T-lymphocytes, most of which express CD4 (helper) antigens (Poulter *et al.*, 1985), surround the interdigitating dendritic macrophage-like HLA-DR⁺ cells. The activated T-lymphocytes produce many lymphokines, including IL-2, IFN- γ and TNF- α , which are the principle regulators of the proliferative immune response. There is growing evidence that such cytokine production in RA is implicated in the pathogenesis of the disease (Brennan *et al.*, 1992; Feldmann *et al.*, 1992).

B-lymphocyte activation is the final immunological event in early rheumatoid synovitis. If the unknown initiating antigen in RA has multiple repeating copies of an epitope, this antigen could bind to B-lymphocyte membrane immunoglobulin receptors, leading to receptor cross-linking and subsequently to B-lymphocytes in an excited state being susceptible to proliferative factors (Paul, 1983). This is a T-cell independent process resulting in polyclonal activation. Alternatively, B-lymphocyte activation may depend on interaction of T-helper cells with B-lymphocytes in an antigen-specific, MHC-restricted manner (Cambier *et al.*, 1985; Howard & Paul, 1983). Once activated, the B-lymphocytes proliferate, and are lifted into an antibody secreting state by B-cell growth and proliferation factors produced by T-lymphocytes.

Rheumatoid factors (RF), reviewed recently by Moore & Dorner, (1993), are autoantibodies primarily directed against epitopes on the Fc fragment of IgG. However, studies have shown that certain isolated RF can also react with nucleosomes, viral proteins and other antigenic material (Williams *et al.*, 1992). Although characteristic of RA they are not disease specific and are associated with inflammatory states in which the immunogenic stimulus persists in the body, and when polyclonal B-lymphocyte activation is present.

1.1.5 EARLY DISEASE MANIFESTATIONS IN THE RHEUMATOID JOINT

Synovial fluid accumulation in inflamed joints is one of the first manifestations of disease activity in RA. In normal joints this fluid is an ultrafiltrate of plasma that passes through the fenestrations along the capillary endothelium. Once in the extracellular space, there is no barrier to fluid collection in the joint cavity, because pressure in the normal joint cavity is negative, compared with that within the postcapillary venules. As synovial fluid accumulates, the intra-articular pressure increases to the point that the synovial blood flow is compromised (Jayson & Dixon, 1970). This increase in pressure, combined with a capillary network that appears inadequate to provide sufficient O₂ to the enormous mass of proliferating cells, leads to acidosis in joints of the most severely affected patients. Large numbers of polymorphonuclear leukocytes (PMNL) accumulate in the synovial fluid, more than one billion may be drawn into the joint cavity of the knee each day in a rheumatoid patient with moderately active disease (Hollingsworth *et al.*, 1967). This occurs by directed migration (chemotaxis) of PMNL to chemoattractants produced and released in the joint space (Snyderman & Lane, 1985). The most important of these and their source are shown in Table 1.1. These substances bind to specific receptors on the PMNL membrane and are necessary and sufficient to activate these cells as they enter the joint space.

TABLE 1.1 *Source Of Some Chemoattractants Produced And Released In The Rheumatoid Joint (from Harris, 1988)*

CHEMOATTRACTANT FACTOR	CELL OR CASCADE SOURCE
Leukotriene B ₄	PMNL membrane phospholipids
Platelet-activating factor	PMNL membrane phospholipids
C5a	From activation of complement
Thrombin	From activation of clotting
Lymphocyte-derived chemotactic factor	Lymphocytes

Activation of PMNL enables them to aggregate and adhere to other cell membranes, to generate oxygen-free radicals which are mediators of tissue injury and disease (Kehrer, 1993), to degranulate and release multiple enzymes including elastase, collagenase and gelatinase, all of which have the ability to degrade collagen, and to generate biologically active products of arachidonic acid by activating the enzymes cyclooxygenase and lipoxygenase. Of the leukotrienes, the PMNL produces only leukotriene B₄ (LTB₄) (Robinson, 1985), the actions of which are shown in Table 1.2 along with the actions

of inflammatory prostaglandins (eg. PGE₂) also produced by activated PMNL in the rheumatoid joint (Mochan *et al.*, 1986).

TABLE 1.2 *Actions Of Leukotrienes And Prostaglandins Produced By Activated PMNL In Rheumatoid Synovium*

ACTION OF MOLECULE IN RHEUMATOID SYNOVIUM
<i>LEUKOTRIENE B₄</i> :
Potent chemoattractant for other neutrophils, eosinophils and macrophages
Enhancement of neutrophil adherence to endothelium
Enhancement of neutrophil aggregation
Enhancement of natural killer cell cytotoxic activity
<i>INFLAMMATORY PROSTAGLANDINS</i> :
Vasodilation
Fever promotion
Sensitization of receptors to pain stimuli
Increase of bone resorption by osteoclasts
Modulation of immune reactions

1.1.6 DESTRUCTION OF CARTILAGE BY PANNUS IN THE RHEUMATOID JOINT

Pannus is a vascular and fibrous granulation tissue arising from the perichondral synovial membrane, which clings tightly to the articular surface of cartilage. The pannus invasion starts from the margin of articular cartilage, which borders the junction between synovial tissue and cartilage. Pannus destroys cartilage either 1) by creeping onto the articular surface of cartilage or 2) by directly penetrating the cartilage and bone, and finally migrating into the medullary space of bone (Fassbender, 1986).

In the early phase of pannus invasion, pannus extends onto the articular surface of cartilage as a layer of morphologically quiescent fibroblast-like cells. As the pannus develops and begins to invade into the cartilage matrix, macrophage-like cells appear just underneath the layer of fibroblast-like cells. In the advanced stage of pannus formation, the pannus becomes thick with blood vessels and cell infiltrates, notably neutrophils and plasma cells. The pathogenesis of pannus formation is reviewed by Shiozawa & Shiozawa, (1988). Either local factors in the cartilage may stimulate pannus formation or the pannus itself possesses an autonomous invasive potential. Implicated in these processes are immune complexes, fibronectin, denuded collagen and the absence of protease inhibitors. Ultimately, the destructive power of pannus

results in unreparable damage to the joint, unless intervention by drugs or spontaneous remission occurs.

1.1.7 CLINICAL FEATURES

No clinical symptoms are produced during the primary phase of RA when a cellular immune response is initiated by an unknown aetiological agent. The symptoms of RA only begin when the production and release of cytokines by macrophages and activated T-lymphocytes occurs, angiogenesis begins in the oedematous synovial membrane and neutrophils are attracted to the joint cavity. The symptoms begin gradually (Fleming *et al.*, 1976) with general fatigue and malaise before stiffness and swelling of one or more of the joints occurs. Once the proliferating synovial membrane has become organised into an invasive front that invades cartilage, tendons and subchondral bone there is a weakening of the joint capsules, muscle wasting and joint instability. These changes in the joint tissues may be visualised by radiography. As the disease progresses the characteristic 'Swan neck' and 'Boutonnière' extreme hand deformities of RA may be seen. Morning stiffness, a sensitive but non-specific symptom of RA, is generated by the development of fibrous adhesions and, along with an increase in extracellular fluid in and around the joint, restricts full joint extension. Extracellular manifestations of RA include the formation of rheumatoid nodules. These are granulomatous swellings found subcutaneously over pressure points, and are characteristically seen at the elbow.

1.1.8 DIAGNOSIS OF RHEUMATOID ARTHRITIS

Due to the heterogenic clinical presentation of RA, there is a fundamental need for the accurate diagnosis of RA if disease management and therapy are to be effective. Criteria proposed for the diagnostic classification of patients with RA were first formulated by a committee of the American Rheumatism Association (ARA) in 1956 and were subsequently revised by the ARA in 1958 (Ropes *et al.*, 1959) in an attempt to improve specificity and simplicity of RA diagnosis. With time and the accumulation of clinical knowledge, the ARA revised the 1958 criteria in 1987 (Arnett *et al.*, 1988) giving rise to a single diagnosis, RA, (as opposed to the old stratification into classic and definite RA), requiring the patient to meet four of the seven criteria shown in Table 1.3. These criteria are constantly being reviewed (Rigby & Wood, 1990) in an attempt to develop simpler and more efficient standards. A set of disease activity measures for use in RA clinical trials have recently been developed (Felson *et al.*, 1993), aiming to improve and standardize the clinical assessment of drugs for RA therapy.

TABLE 1.3 1987 Criteria For Diagnosis of Rheumatoid Arthritis (from Arnett *et al.*, 1988)

CRITERION
Morning stiffness for at least 1 hour
Swelling of the soft tissue around at least 3 of the 14 designated joint groups
Swelling of a hand or wrist joint
Rheumatoid nodules
Symmetrical arthritis
Rheumatoid factor
Radiographic evidence of erosions in the joints of the hands or wrist

1.1.9 ANIMAL MODELS OF RHEUMATOID ARTHRITIS

Animal models of RA have provided useful systems for the characterization of the immunopathogenic mechanisms of chronic inflammatory synovitis and for the testing of anti-arthritic agents. Ideally, an animal model should have clinical and pathological features very similar to those seen in RA. The model should show a susceptibility to disease induction, a reliable onset, severity and incidence of disease, with an accelerated disease activity and immune function similar to RA which responds to drugs clinically effective in RA therapy, and the animal model should ideally have parameters of disease activity which are readily measurable.

Various methods have been applied to induce in animals experimental models of arthritis. Antigen-induced arthritis has been the most commonly studied animal model of inflammatory arthritis since Dumonde & Glynn, (1962), reported the induction of chronic synovitis in sensitized rabbits by the intra-articular injection of fibrin. Various antigens are capable of inducing acute and chronic synovitis with pannus formation, synovial hypertrophy, hyperplasia and infiltration of neutrophils, plasma cells, macrophages and lymphocytes.

Currently, heat-killed *Mycobacterium* emulsified in incomplete Freund's adjuvant (Pearson, 1956), type II collagen (Trentham *et al.*, 1977), and bacterial peptidoglycans from Group A *Streptococcus* and *Lactobacillus casei* (Cromartie *et al.*, 1977), are the three most frequently studied antigenic agents used to induce arthritis in small animals. One of the major disadvantages encountered in all these types of models is due to the size of the animal joints. It is technically difficult to administer inflammatory stimuli

and collect sufficient synovial fluid for analysis without traumatizing the joint, which may itself lead to inflammation.

In 1953, Selye observed that the subcutaneous injection of air into rats resulted in a pouch granuloma. The structure and cell composition of the air-pouch lining has been shown to be very similar to that of the synovial lining (Edwards *et al.*, 1981). Moreover, injection of various stimuli including aggregated gamma-globulin (Kowanko *et al.*, 1986), carrageenan (Sedgwick *et al.*, 1985), and zymosan (Konno & Tsurufuji, 1983), results in an inflammatory response with histological similarities to acute and chronic synovitis. This model has the significant advantage of overcoming the technical problems encountered with conventional antigen-induced arthritis models. However, the extent of its similarity to normal synovium in terms of it being non-weight bearing and having no cartilage is, as yet, unclear. This model does allow though for investigations into cell trafficking and cartilage degradation during inflammation and drug therapy to be explored.

1.2 THE TREATMENT OF RHEUMATOID ARTHRITIS

Due to the clinical heterogeneity of RA it is not possible to describe a therapeutic regime which is applicable to all RA patients. Indeed, the treatment of RA continues to be one of the most challenging problems in clinical medicine today. The major treatment goals for RA are; 1) control of inflammation and pain, 2) restoration and maintenance of optimal function, 3) suppression of constitutional and extra-articular disease, and 4) avoidance of medicament toxicity (Dugowson & Gilliland, 1986). In achieving these goals it is necessary to carefully assess the course and prognosis of each patient individually, and the attendant risks and benefits of the available drugs. Generally, the treatment of RA is in a pyramid format, beginning with the use of non-steroidal anti-inflammatory drugs (NSAIDs) and followed by disease-modifying anti-rheumatic drugs (DMARDs) as the disease progresses. In cases of severe destructive RA, cytotoxic agents and corticosteroids may be tried as well as surgical intervention therapy, with more novel therapies being employed as a last resort to stem the progression of the disease.

1.2.1 NON-STEROIDAL ANTI-INFLAMMATORY DRUGS

NSAIDs are the first-line drugs of RA therapy. They have a rapid onset of action and improve pain, swelling and morning stiffness. However, they have no proven ability

to affect the course of the basic disease processes in RA and therefore do not afford protection against tissue or joint injuries.

Five major chemical groups of non-steroidals are commonly used, namely salicylates, e.g. Aspirin; propionic acid derivatives, e.g. Ibuprofen and Naproxen; indoleacetic acid derivatives e.g. Indomethacin and Sulindac; mefenamic acid derivatives e.g. Meclofenamate; and oxicam derivatives e.g. Piroxicam. All have a similar therapeutic effect, being antipyretic, analgesic and anti-inflammatory, which is a result of their inhibition of prostaglandin synthesis (Abramson & Weissmann, 1989). There is no reliable method for determining the best non-steroidal for a given patient in terms of efficacy and toxicity except by trial and error. Once an effective drug has been identified there is only a small likelihood of another being more effective, and it is then the effective drug's toxicity and tolerability which then determines whether another drug should be tried.

Gastrointestinal side effects of NSAIDs vary greatly and include vomiting, nausea, anorexia, diarrhoea, heartburn and abdominal pain. Gastric irritation arises from drug contact with the gastric mucosa leading to inhibition of prostaglandin formation in the gastric wall. This predisposes patients to the symptoms of gastric and peptic ulcer disease (Simon, 1990).

1.2.2 DISEASE-MODIFYING ANTI-RHEUMATIC DRUGS

DMARDs have been reviewed by Rainsford, (1990), and constitute a diverse group of compounds which share a common pattern of clinical response. After weeks or months of administration the subtle onset of clinical benefit may occur and, with continued administration, complete suppression of some or all of the disease manifestations of RA can occur in some patients. They have minimal non-specific anti-inflammatory effects unlike the NSAIDs and do not induce systemic immunosuppression like cytotoxic agents. Disease-modifying drugs include gold compounds, d-penicillamine and antimalarials, each of which was developed for other purposes and then applied to rheumatic diseases as an afterthought.

1.2.2.1 GOLD COMPOUNDS

The anti-rheumatic properties of gold were first advocated in the late 1920s and, by 1935, Forestier had established the basis for the modern clinical use of gold

compounds. Intramuscular injections of aqueous sodium aurothiomalate and orally dosed Auranofin are the most common preparations available in the UK.

Gold therapy offers a 30-40% probability of complete control of inflammation and a 60-80% probability of useful clinical improvement (Sambrook *et al.*, 1982). The mode of action of gold complexes has been reviewed by Haynes & Whitehouse, (1989). Much still remains to be understood, including the slow onset of beneficial clinical effects of gold therapy and the major variations between patient response. The prolonged duration of action of gold compounds is related to the level of gold retained within the body. The major limitations to the use of gold therapy are toxicity and the cost of patient monitoring. The side effects are well documented (Smith & Brooks, 1984) and include dermatitis, renal toxicity and hematological abnormalities. This necessitates routine patient monitoring with physical examinations and peripheral blood counts, as well as testing for hematuria and proteinuria.

1.2.2.2 D-PENICILLAMINE

D-penicillamine was originally developed as a copper-chelating agent for patients with Wilson's disease. Based on the knowledge that it could also dissociate macroglobulins, Dresner & Trombly, (1960), showed that D-penicillamine could also dissociate RF *in vitro*. Although clinical trials have shown it to be comparable to other second line drugs including gold compounds (Howard-Lock *et al.*, 1986), its use has been severely limited by a multiplicity of toxicities including cutaneous, renal and hematological complications, along with dysgeusia, the complete loss of taste perception, and the possibility of the activation of other autoimmune conditions.

1.2.2.3 ANTIMALARIALS

Chloroquine and subsequently hydroxychloroquine have been used in RA therapy since the 1950s (Haydu, 1953). Although their mechanisms of action remain unclear, their therapeutic effect is evidenced by improvement in clinical and laboratory criteria of disease activity (Zvaifler, 1988). The most serious toxicity of antimalarials involves ocular complications, with regular slit-lamp examinations every six months being required.

1.2.2.4 METHOTREXATE

Methotrexate, reviewed by Grosflam & Weinblatt, (1991), is widely accepted by rheumatologists as an alternative to gold therapy, and has the advantage that low dose, weekly oral or parenteral administration is the most rapidly acting DMARD, often effective in the first month of therapy. The mechanism of action has yet to be clearly defined. The toxicity of methotrexate includes bone marrow suppression and liver dysfunction, with liver biopsies routinely needing to be taken.

1.2.2.5 CYCLOSPORIN A

Cyclosporin A acts specifically and reversibly on lymphocytes, in particular T-helper cells, producing selective suppression of cell-mediated immunity and the inhibition of several cytokines, most notably IL-2 and IFN- γ . In light of this, the rationale for a possible role for cyclosporin A in the treatment of RA is justified. Oral cyclosporin A therapy is reported to be at least as effective as azathioprine, chloroquine, methotrexate or d-penicillamine in patients with severe RA (Faulds *et al.*, 1993). It is recommended that low doses of cyclosporin A are initially used as patients seem particularly susceptible to the renal adverse effects of this drug.

1.2.3 CYTOTOXIC THERAPY

Due to the failure of the available DMARDs to stop the progression of disease in some RA patients, cyclophosphamide and azathioprine have been studied in the rheumatoid population and have been shown to be effective (Fox & McCune, 1989). However, their potential for toxicity, including bone-marrow depression, teratogenicity, induction of malignancy and the increased threat of infection, have limited their clinical application. Cytotoxic drugs are therefore only used as a last resort for RA patients who have active, erosive disease, and who have failed to respond to DMARDs.

1.2.4 CORTICOSTEROID THERAPY

The insight which initiated the course of events leading to the use of corticosteroids as a treatment for RA occurred in 1929 (Hench, 1952). In 1948, the first rheumatoid patient was treated with cortisone leading to a dramatic improvement in clinical symptoms (Hench *et al.*, 1949) and a Nobel Prize in Medicine and Physiology for Hench. With their commercial production corticosteroids became widely prescribed for

RA. However, many reports suggested there was no disease-modifying action of corticosteroids and, coupled with the appreciation of the numerous complications of corticosteroid therapy (Von Eickstedt & Elsasser, 1988), including peptic ulceration, hypertension, sodium and water retention, and osteoporosis to name but a few, corticosteroids were placed at the apex of the treatment pyramid of RA and were considered to be agents of last resort. Re-examination of the literature (George & Kirwan, 1990; Weiss, 1989), however, has found little justification for the relegation of corticosteroids to the status of non-disease modifying agents, and there is support for the concept of a disease-modifying role of corticosteroids (Empire Rheumatism Council, 1957; Harris *et al.*, 1983; Million *et al.*, 1984).

1.2.4.1 CLINICAL PHARMACOLOGY AND PHARMACOKINETICS

A comparison of commonly used glucocorticoids in RA therapy is shown in Table 1.4. Orally dosed hydrocortisone is readily absorbed across the gastrointestinal tract and peak blood concentrations are achieved in 90 minutes. The plasma half-life of hydrocortisone is about 90 minutes, but the biological half-life in terms of anti-inflammatory action in tissues is longer (8-12 hours) (George & Kirwan, 1990). In normal or low plasma concentrations, 90-95% of hydrocortisone is bound to plasma proteins. About 10% is bound to albumin and about 80% to transcortin, an α_2 macroglobulin with a low capacity, but high affinity for hydrocortisone. At higher concentrations there is an increase in albumin-bound and free hydrocortisone, the remaining unbound hydrocortisone existing in equilibrium with bound hydrocortisone (Baxter & Forsham, 1972).

Pulsed intravenous corticosteroid therapy involves the intravenous infusion of a large dose of corticosteroid (usually around 1 g of methylprednisolone) over a short time - around 30 minutes (Weiss, 1989). A variety of regimes are currently used, but most entail a course of three pulses on alternate days, followed by a resting phase of around six weeks. This form of treatment was initiated in renal transplant recipients, but the observation was made in patients with renal disorders and RA, that synovial inflammation responded rapidly and for prolonged periods with intravenous therapy. Despite a number of controlled studies, however, opinion regarding its usefulness remains divided (Kimberly, 1991). Adverse effects of this type of treatment regime include sudden death, severe infections, gastrointestinal bleeding and visual disturbance, and, as such, pulsed therapy is only used as an acute therapeutic intervention.

TABLE 1.4 Comparison Of Commonly Used Glucocorticoids In RA Therapy (from George & Kirwan, 1990)

DURATION OF ACTION	GLUCOCORTICOID	EQUIVALENT ORAL OR IV DOSES (mg) FOR SIMILAR PHARMACOLOGICAL EFFECT
<i>SHORT</i> $t_{0.5}$ 8-12 hours	Cortisone	25
	Hydrocortisone	20
<i>INTERMEDIATE</i> $t_{0.5}$ 12-36 hours	Prednisone	5
	Prednisolone	5
	Methyl prednisolone	4
	Triamcinolone	4
<i>LONG</i> $t_{0.5}$ 36-72 hours	Paramethasone	2
	Dexamethasone	0.75
	Betamethasone	0.6

Intra-articular injection of corticosteroids into inflamed rheumatoid joints was devised by Hollander *et al.*, (1951) and can, in some cases, provide prolonged relief from pain and swelling and markedly improve joint mobility and function. Hydrocortisone as sodium phosphate or succinate are usually employed (up to 200 mg depending on the joint size), whereas the hydrocortisone acetate is used in doses of 5-50 mg.

The duration of corticosteroid action within the joint is reported to vary inversely with the solubility of the agent being used (Gray *et al.*, 1981). After injection into the synovial joint cavity hydrocortisone is rapidly cleared in a biexponential fashion with $t_{0.5\alpha}$ of 22 minutes and $t_{0.5\beta}$ of 150-250 minutes (Winter *et al.*, 1967). Wallis & Simkin, (1983), suggest that the initial phase relates to the intra-articular distribution and the second, slower phase reflects true trans-synovial exchange.

Disadvantages of this route of drug administration include the possibility of infection, although this is rare if aseptic techniques are employed, acute crystal synovitis which is self-limiting and usually resolves within 48 hours (Gray *et al.*, 1981), and patient discomfort during the injection process.

1.2.4.2 MODE OF ACTION

Pharmacological doses of glucocorticoids profoundly suppress a wide variety of non-specific inflammatory responses and specific immunological processes (George & Kirwan, 1990), and are summarized in Table 1.5. Their effect is achieved by controlling the rate of synthesis of mRNA and proteins. Free hydrocortisone diffuses into individual cells, where specific receptor proteins are found in the cytoplasm of glucocorticoid-responsive tissues. The steroid and receptor form a complex that undergoes a conformational change and leaves the cytoplasm, moving to the nucleus. Here, the complex binds reversibly to specific sites on the chromatin, thereby increasing or reducing the synthesis of the corresponding mRNA and subsequent protein synthesis.

TABLE 1.5 *Some Pharmacological Actions Of Glucocorticoids (adapted from George & Kirwan, 1990)*

PHARMACOLOGICAL ACTIONS OF GLUCOCORTICIDS
Increased synthesis of lipocortin and subsequent inhibition of phospholipase A ₂
Reduced production of cytokines and inflammatory enzymes
Alteration in B- and T-lymphocyte functions
Reduction in Fc receptor expression
Changes in white cell traffic

Glucocorticoids especially increase the rate of synthesis of lipocortin. The anti-inflammatory effect of lipocortin is mediated through its inhibition of the enzyme phospholipase A₂ (Figure 1.2). Arachidonic acid release from the membrane phospholipids is thus blocked with the production of pro-inflammatory substances *via* cyclooxygenase and lipoxygenase reduced.

Glucocorticoids also inhibit the inflammatory process by reducing the formation of various vasoactive chemicals released during inflammation including kinins, histamine, lysosomal enzymes, eicosanoids, cytokines and complement system components. The humoural immune response may also be modulated by glucocorticoids. Cell mediated responses are not inhibited *per se* but their manifestations are prevented. These are summarized in Table 1.6.

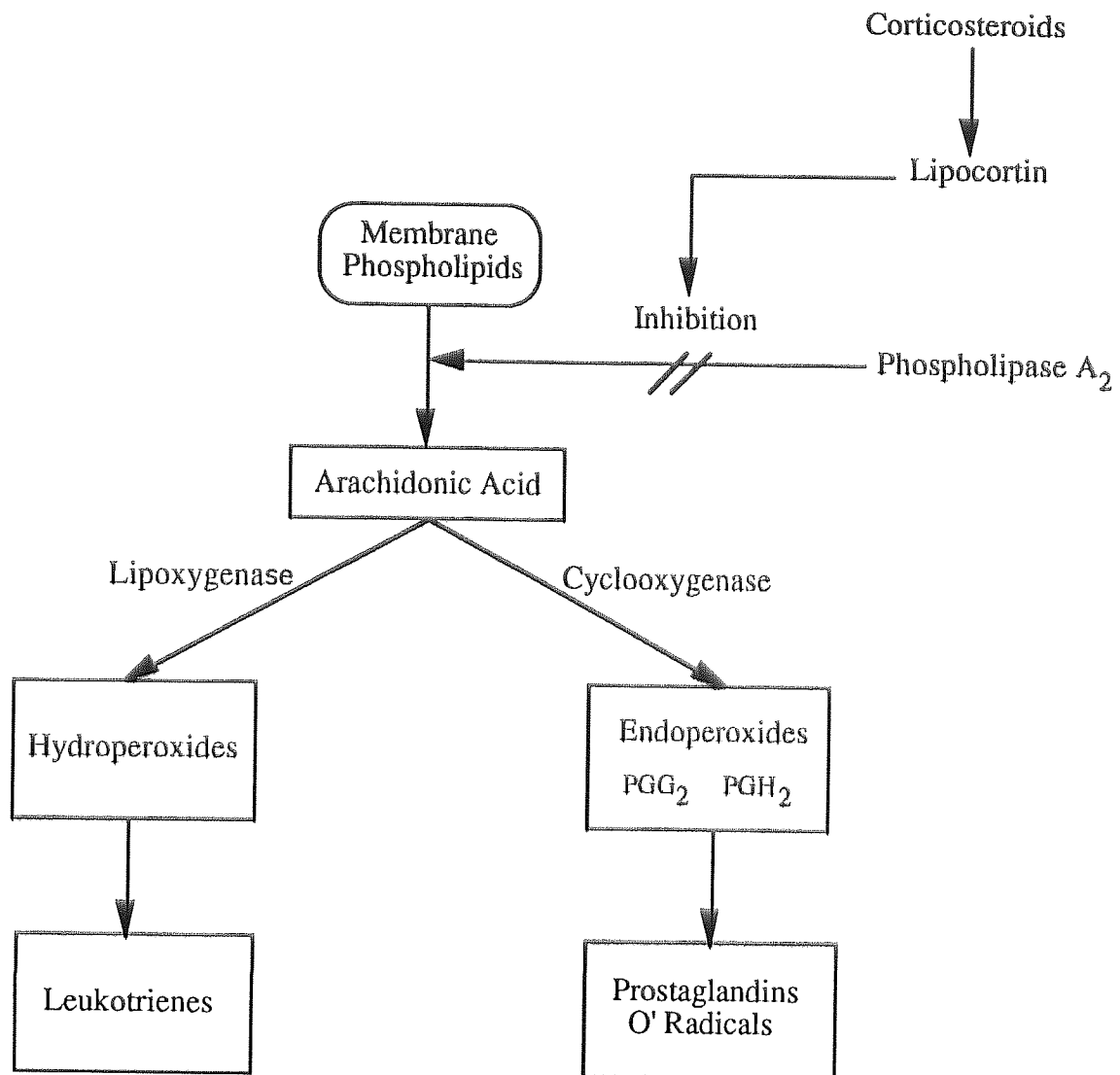


FIGURE 1.2 *Proposed Mechanism Of Glucocorticoid Inhibition Of Pro-Inflammatory Mediators (from George & Kirwan, 1990)*

Glucocorticoids induce the synthesis of lipocortin, which inhibits the enzyme phospholipase A₂. Arachidonic acid release from membrane phospholipids is thus blocked. The production of pro-inflammatory substances via cyclooxygenase and lipoxygenase is thus reduced.

Another mode of action has recently been proposed for glucocorticoid anti-inflammatory activity by Tessier *et al.*, (1993). As described in Section 1.1.4, accumulation of leukocytes at the site of inflammation is controlled to a large extent by the expression of adhesion molecules and their corresponding counter-receptors on the surface of leukocytes and target cells, such as endothelial cells and connective tissue fibroblasts. The expression of adhesion molecules on the cell surface is induced by a variety of cytokines including TNF- α . Since glucocorticoids have been shown to

inhibit TNF- α production (Culpepper & Lee, 1987), the expression of the adhesion molecule intercellular adhesion molecule-1, (ICAM-1), on the synovial fibroblasts is down regulated, and therefore leukocyte infiltration into the site of inflammation may be reduced (Tessier *et al.*, 1993).

TABLE 1.6 *Pharmacological Effects Of Glucocorticoids On The Cellular Components Of The Immune System*

PHARMACOLOGICAL EFFECT OF GLUCOCORTICOID ACTION
<p><i>MONOCYTES/MACROPHAGES</i></p> <p>Redistribution from peripheral blood to other lymphoid compartments.</p> <p>Alteration in cell surface protein expression (decreased Fc and C3 receptors and HLA-DR antigens).</p> <p>Inhibition of cytokine secretion including IL-1 and TNF-α.</p> <p>Inhibition of proteolytic and inflammatory protein synthesis and function (collagenase, stromelysin, lipoxygenase, cyclooxygenase).</p> <p>Inhibition of chemotaxis, phagocytosis and pinocytosis.</p> <p>Inhibition of bacterial and fungicidal activities.</p>
<p><i>T-LYMPHOCYTES</i></p> <p>Redistribution from peripheral blood to other lymphoid compartments.</p> <p>Inhibition of lymphokine production including IL-2.</p> <p>Inhibition of suppressor T-lymphocytes.</p>
<p><i>B-LYMPHOCYTES</i></p> <p>Redistribution from peripheral blood to other lymphoid compartments.</p> <p>Inhibition of activation, proliferation and immunoglobulin production.</p>

1.2.5 SURGICAL TREATMENT

Synovectomy involves the removal of the synovial lining from the affected joint, removing the diseased synovium and pannus, and thus preventing cartilage degradation due to lysosomal enzymes released by the invading inflammatory tissue. Patients with severe pain and joint dysfunction may undergo a prosthetic joint replacement. The refinement of prosthetic arthroplasties over the past two decades has dramatically improved surgical rehabilitation of patients with severe disabilities from chronic arthritis. The success rate is high for hip and knee operations (Gross *et al.*, 1991) and is increasing for other joints such as the elbow and ankle.

1.2.6 IMMUNOTHERAPY

As the understanding of the cellular pathology of RA advances a more rational approach to its treatment has developed whereby targets for selective therapy have been pursued. As described in Section 1.1.4, T-lymphocytes appear to play a major role in the immunopathogenesis of RA. Therapies which selectively repress T-cell function such as thoracic duct drainage (Paulus *et al.*, 1977), lymphapheresis (Karsh *et al.*, 1981), total lymphoid irradiation (Brahm *et al.*, 1984), and antithymocyte globulin administration (Shmerling & Trentham, 1989), have ameliorated disease activity in patients with RA. However, although such treatments affect a defined limb of the immune response, they are relatively non-specific in their action and are complicated by side effects.

One of the least toxic and potentially most effective approaches for specific immunological intervention in the disease process is T-cell vaccination (TCV). This procedure has been shown not only to be effective in inducing resistance to the future development of experimental autoimmune arthritis (Cohen & Weiner, 1988), but also to be effective in inducing lasting remission of already established disease (Lider *et al.*, 1987). There are several possible targets for TCV, reviewed by Willoughby & Seed, (1992), and include: i) antagonism of the MHC class II receptor, ii) antagonism of the T-lymphocyte receptor (TCR) or vaccination against TCR-bearing CD4⁺ cells, iii) induction of autoreactive CD8⁺ cells, and iv) modulation of the expression of the endogenous antigen of inflammation.

Work on induced autoimmune diseases in experimental animals has demonstrated that specific monoclonal antibodies, raised against various antigens, have great therapeutic efficiency even at advanced stages of the disease (Acha-Orbea *et al.*, 1988). T-cell membrane molecules including the TCR, the associated CD3 molecules, the IL-2 receptors, the MHC-class directing molecules CD4 and CD8, and defined adhesion molecules on endothelial cells, have all been targeted as potential sites for immune intervention with monoclonal antibodies (Breedveld & De Vries, 1990).

1.2.7 LASER THERAPY

The ability of laser beams to cut, cauterize and destroy tissue is well known. Less well known is that low intensity laser irradiation can non-destructively modulate biological processes, including having a multitude of effects on immunological cell function. Well controlled clinical trials using low intensity laser therapy for treatment of RA have

been carried out and have been recently reviewed by Basford, (1993). Although many of these trials have reported that RA patients have responded well to laser therapy, others do not find it helpful, and even in the positive reports the benefits may be marginal. With continuing well controlled trials in progress it is hoped that treatment effectiveness will be clarified within a few years.

1.2.8 DIETARY THERAPY

The idea that dietary manipulation may alter the symptoms of RA is part of the folklore of the disease. However, until recently there has been little scientific support for these claims. Since dietary treatment is safe, and may reduce or avoid the need for drugs, it is an appealing therapy to most patients.

Dietary therapy for RA may be divided into two subtypes: supplementation therapy, in which substances are added to the diet, and elimination therapy, in which foods are removed from the diet. Among dietary supplements, the best studied are fish oil, evening primrose oil, New Zealand green-lipped mussel and selenium, but vitamins, herbs, kelp, royal jelly, ginseng, cider vinegar and honey are also popular. The dietary elimination programme falls into three main parts; the elimination phase, the re-introduction phase and the double-blind challenge, whereby the food producing the symptoms of RA is identified and confirmed. Darlington & Ramsey, (1993) have extensively reviewed the available literature on both these types of dietary therapy, and although much scientific research remains to be done the potential for benefit is great.

1.3 THE NEED FOR GREATER SITE-SPECIFICITY

It would be of considerable advantage for RA drug therapy if drugs administered to the body would have the ability to reach their sites of action in high concentrations with little or none reaching extraneous sites. This would implement a lower degree of possible side effects from the currently available drugs for RA therapy. However, it should be kept in mind that not only should the drug reach the target tissue site, the cells at this site, and possibly structures within the cell, but the drug should also be retained at the tissue site for a suitable time period and at a suitable level in order to exert its action. The prime elements to be considered in site-specific drug delivery, besides the importance of the disease and the drug, are access, retention and timing (Tomlinson, 1987).

1.3.1 LOCAL ADMINISTRATION

While it is clear that local administration of drug to a target site is not a particularly sophisticated form of drug targeting, intra-articular (i.a.) injection of anti-inflammatory drugs in the form of corticosteroids has been part of RA therapy since their introduction in 1951 (Hollander *et al.*, 1951). Numerous long-acting steroid ester preparations have been developed to enhance anti-inflammatory activity and reduce hormonal side-effects. Duration of drug action is related to insolubility and, therefore, prolonged retention at the site of injection (Gray *et al.*, 1981). This is discussed in greater detail in Section 1.2.4.1.

In order to enhance the retention of drug within the synovial cavity, drugs have been incorporated into liposomes and monolithic particles which may be injected into the joint. The persistence of such particles within the joint is related to their size, with larger particles (7-15 μm in diameter) being retained for longer periods (Noble *et al.*, 1983). There is also evidence that particulates may be phagocytosed by the fixed macrophages within the synovium (Ratcliffe *et al.*, 1984). The intra-articular administration of liposomes containing cortisol palmitate has been used to treat experimental arthritis in the knee joints of rabbits at relatively low doses of the drug (Dingle *et al.*, 1978). Recently, work by Foong & Green, (1993), has shown the potential of the i.a. administration of liposomal methotrexate as a local drug delivery system to reduce the adverse systemic effects of i.a. methotrexate therapy.

Although the suppression of inflammatory symptoms by the i.a. injection of drugs has been confirmed in numerous studies (Gray *et al.*, 1981), it is clearly impossible to inject more than a few joints, and the same joint may only be injected for a limited number of times. Patients with many diseased joints may, therefore, be better treated with systemic drug preparations.

1.3.2 SITE-SPECIFIC DRUG DELIVERY

The concept of site-specific drug delivery is an old one, first being suggested by Erlich in the late nineteenth century (Baumler, 1984). However, over the past two decades full consideration has been given to the ways in which this might be achieved in practice, due largely to the advent of new biosciences (Tomlinson, 1986).

Site-specific drug delivery may be achieved by using carrier systems, where reliance is placed on exploiting both the innate pathway(s) that these carriers have, and the

protection that they can afford to drugs during transit through the body. Such carrier systems can be broadly categorized as soluble macromolecular drug conjugates, particulate delivery systems and cellular drug carriers (reviewed by Friend & Pangburn, 1987). For the purpose of this work, only particulate drug carrier systems will be discussed.

1.4 PARTICULATE DRUG CARRIER SYSTEMS

Several names have been given to particulate drug carrier systems including microparticles (microspheres and microcapsules), nanoparticles (nanospheres and nanocapsules) and microsponges. Davis & Illum, (1989), classified nanoparticles as those particles below 500 nm. Other groups (Arshady, 1993; Donbrow, 1992; Kreuter, 1992) classify nanoparticles as particulate carriers of a size range up to 1000 nm. Strictly speaking, microspheres and nanospheres should consist of a matrix and perhaps be solid in nature. A microsphere has its drug dispersed throughout the particle; *ie.*, the internal structure is a matrix of drug and polymeric excipient (Tomlinson, 1983). Microcapsules and nanocapsules are similar in many respects to microspheres and nanospheres respectively, but here the drug is loaded centrally within the particle, where it is encased within a polymeric membrane (Donbrow, 1992).

A wide variety of base materials have been used in microparticle/nanoparticle manufacture, with examples from the literature shown in Table 1.7. Microparticles may function as carriers for drugs to desired regions in the body and as depots from which the drugs are released at the site of action in a controlled manner (Oppenheim, 1988). Depending on size and the nature of materials used, microparticles can serve as dosage forms for oral, parenteral and other routes. The choice of material for the microparticle will be influenced by toxicity considerations that include the immune response. In oral dosage forms, many materials; insoluble, biodegradable, or non-degradable, may be employed, whereas in parenteral dosage forms, biodegradable, or resorbable materials with high biocompatibility are needed (Juni & Nakano, 1992). A brief introduction to two microspheres drug delivery systems will be considered in more detail in this thesis; namely albumin microspheres and poly(lactic acid), (PLA), microspheres.

TABLE 1.7 *Some Examples Of Microsphere/Nanosphere Drug Delivery Systems Described In The Literature (adapted from Davis & Illum, 1989)*

MATERIAL	DRUG	REFERENCE
Albumin	Aminophylline	Tripathi & Singh, (1992)
Ethyl Cellulose	Cisplatin	Okamoto <i>et al.</i> , (1986)
Eudragit®	Indomethacin	Bodmeier & Chen, (1989)
Fibrinogen	5-fluorouracil	Miyazaki <i>et al.</i> , (1986)
Gelatin	5-fluorouracil	Oppenheim <i>et al.</i> , (1984)
Polyanhydrides	Various proteins	Tabata <i>et al.</i> , (1993)
Poly(butylcyanoacrylate)	Progesterone	Li <i>et al.</i> , (1986)
Poly(hydroxybutyric acid)	Sulphamethizole	Regina Brophy & Deasy, (1986)
Poly(lactide)	Gentamicin	Sampath <i>et al.</i> , (1992)
Poly(lactide-co-glycolide)	Timolol maleate	Sturesson <i>et al.</i> , (1993)
Poly(methylmethacrylate)	Indomethacin	Bodmeier & Chen, (1989)
Starch	Insulin	Gizurason & Bechgaard, (1991)

1.4.1 ALBUMIN MICROSPHERES

Albumin microspheres were first prepared for the detection of abnormalities in the mononuclear phagocyte system (MPS) and blood circulation (Rhodes *et al.*, 1969; Rhodes *et al.*, 1968; Scheffel *et al.*, 1972; Zolle *et al.*, 1970). Kramer (1974) was the first to suggest the use of albumin microspheres as vehicles for achieving site-specificity in drug delivery. This idea was then practically demonstrated by Sugibayashi and co-workers (Sugibayashi *et al.*, 1977). Since this time, several workers have forwarded the development, design, and *in vitro* and *in vivo* evaluation of this delivery device.

The suitability of albumin for use as a drug carrier comes from its inherent properties. Soluble human albumin in blood plasma is a natural circulatory drug carrier. Equilibrium binding of albumin to drug depends primarily on hydrophobic and electrostatic interactions (Koch-Weser & Sellers, 1976), thus eliminating the need for the covalent attachment of albumin to drug, and thereby facilitating drug release.

Toxicological studies have shown albumin microspheres to be biocompatible (Ratcliffe *et al.*, 1984; Royer *et al.*, 1983) and it has been reported that albumin microspheres are not antigenic in species from which the albumin has been isolated for their synthesis (Rhodes *et al.*, 1969). Albumin microspheres are biodegradable, being susceptible to enzymatic attack both *in vitro* and *in vivo* by proteases (Lee *et al.*, 1981; Yapel, 1985), a process which is dependent on the degree of microsphere stabilization in the manufacturing process discussed below. The greater the extent of stabilization, the longer the biodegradation time (Lee *et al.*, 1981). A recent study by Schäfer *et al.*, (1994), has investigated the biodegradation of albumin microspheres by macrophages *in vitro*, with their degradation completed within seven days of macrophage phagocytosis.

Several methods have been reported in the literature for the synthesis of albumin microspheres (reviewed by Gupta & Hung, 1989a) (see Figure 1.3). Most methods involve the application of emulsion and suspension technology. Routine synthesis of particles utilizes an aqueous solution of protein, a therapeutic agent at its saturation solubility, and a suitable vegetable oil to form a w/o emulsion. The emulsion is stabilized after transferring to an oil medium, so as to harden and maintain the rigidity, and to subsequently isolate, the protein spheres. Two methods are popular for the stabilization process: (1) thermal denaturation at temperatures between 100°C - 180°C and (2) chemical hardening using a suitable cross-linking agent such as formaldehyde, glutaraldehyde or 2,3-butanedione. These procedures are reviewed by Yapel, (1985). During the heating process water is removed from, and ultimately insolubilizes, the microspheres *via* probable scrambling of disulfide bonds and the formation of lysinoalanine cross-links (Arshady, 1990). Cross-linking of albumin by glutaraldehyde is a complex reaction and the chemical modification has yet to be clearly defined. The various mechanisms which have been proposed indicate that condensation reactions and lysine side chains are involved (Sokoloski & Royer, 1984).

In view of the fact that albumin microspheres are intended either for diagnostic or drug delivery purposes, their size and surface characteristics are of the utmost importance. Since a large number of different suspension systems and crosslinking processes have been reported in the literature, the effects of various parameters on particle size and other particle characteristics have been systematically investigated by several groups (Gallo *et al.*, 1984; Gupta *et al.*, 1989c; Torrado *et al.*, 1989). Variables in the manufacturing process which influence particle size and size distribution include albumin concentration, emulsification time, power of emulsification, aqueous-to-non-aqueous phase volumes, stirring rate, heat stabilization time, concentration of cross-

linking agent and type of non-aqueous phase. By altering these variables it is possible to produce albumin microspheres with diameters ranging from nanometers up to millimeters.

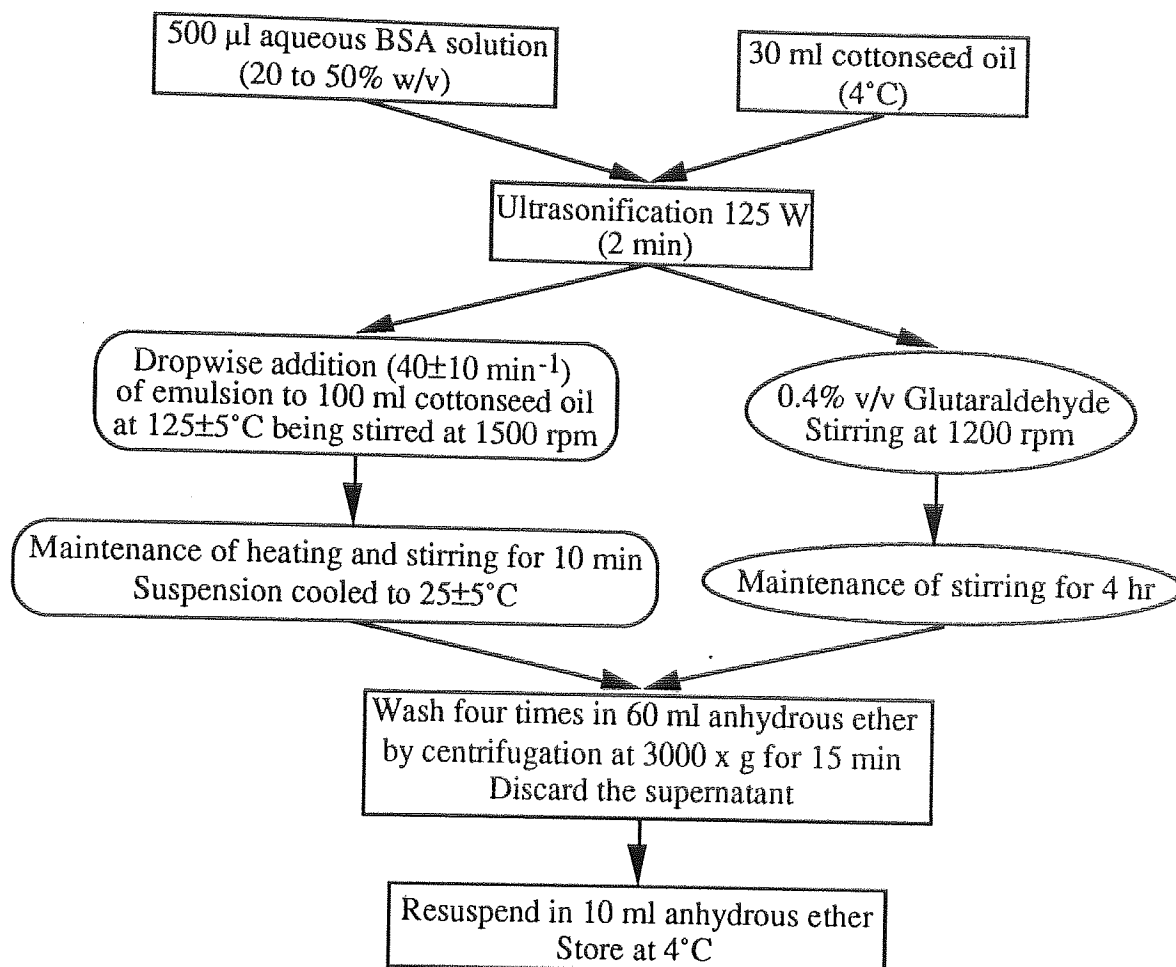


FIGURE 1.3 *Schematic Representation Of The Steps Followed During The Manufacture Of Thermal-Denatured And Chemically Cross-Linked Albumin Microspheres (adapted from the methods reported by Gallo et al., 1984; Yapel, 1985)*

For albumin microspheres to be an effective drug delivery system and alleviate diseases, the minimum effective concentration of medicament must be attained at the target site (Friend & Pangburn, 1987), and this depends on their drug content and rate of drug release (Davis, 1986; Poste & Kirsh, 1983; Poznansky and Juliano, 1984). An increase in drug entrapment in albumin microspheres would reduce the amount of carrier required to deliver a specified dose of the drug, and hence improve its target tissue uptake at the capillary bed (Oppenheim *et al.*, 1984; Willmott *et al.*, 1985). Therefore, constant efforts have been made to design albumin microspheres with high levels of drug entrapment. Inclusion of a therapeutic agent into albumin microspheres

can be attained by two methods: (1) drug addition in the aqueous phase before emulsification with the oil phase (Gupta *et al.*, 1989c; 1986a; 1986b; Sugibayashi *et al.*, 1977); and (2) drug addition and equilibrium with a suspension of pre-formed placebo albumin microspheres (Benita *et al.*, 1984b; Kramer, 1974). Implicitly, the first method is the most commonly adopted technique and it allows the mechanical entrapment of drug to a varying degree of depth in the particles (Gupta *et al.*, 1986a). In contrast to this, addition of the drug to the pre-formed particles allows only its physical adsorption, the degree of which depends upon the number of binding groups available on the particle surface (Royer *et al.*, 1983). In general, comparatively less amount of drug is associated with the particles by this technique.

Albumin microspheres have been prepared incorporating an extensive variety of therapeutic and diagnostic agents (reviewed by Gupta & Hung, 1989a, 1989b). They have been proposed as drug delivery systems for targeting to various organs and tissues including the lungs (Pande *et al.*, 1991; Tripathi & Singh, 1992), liver (Morimoto *et al.*, 1981), tumours (Goldberg *et al.*, 1992), articular joints (Ratcliffe *et al.*, 1987), and to sites of inflammation (Lewis *et al.*, 1992). The anatomical barriers that have to be overcome when targeting drugs to these sites are discussed in Section 1.5.

1.4.2 POLYLACTIC ACID MICROSPHERES

PLA is a non-toxic, biodegradable polymer which is well tolerated by the human body (Kulkarni, 1966). PLA degrades by undergoing random, non-enzymatic hydrolytic de-esterification to form lactic acid and water, which are normal metabolic compounds utilised in the Krebs cycle, producing carbon dioxide and water (DeLuca *et al.*, 1987; Tice & Cowsar, 1984). Due to these properties, PLA has been used for a long time in a variety of applications such as absorbable sutures (Wise *et al.*, 1979), vascular prostheses (Albizzati, 1989), and fracture fixation (Bos *et al.*, 1991). PLA has also been used in the development of microparticulate drug delivery systems (reviewed by Conti *et al.*, 1992).

Low molecular weight PLA polymers (<3000) are produced by the direct condensation of lactic acid (Hutchinson & Furr, 1985; Kulkarni, 1971), (Figure 1.4). High molecular weight products are formed by ring-opening polymerizations of the corresponding cyclic dimer (Figure 1.4), catalysed by zinc oxide or tetraphenyl tin (Hutchinson and Furr, 1985; Kulkarni, 1966; 1971; Marcott & Goosen, 1989). Polymers with a particular molecular weight can be manufactured by the choice of

polymerization conditions (Marcotte & Goosen, 1989). Lactide is the dimer of lactic acid and so polymers synthesized from these are called poly(lactides). Because lactic acid is optically active, the homopolymer formed may be poly(D-lactide), poly(L-lactide) and the racemic poly(DL-lactide) (Rosen *et al.*, 1988). Usually, poly(L-lactide) and poly(DL-lactide) have been used for controlled delivery release systems (Tice & Cowsar, 1984). These polymers are insoluble in water, ethanol and methanol, and soluble in some organic solvents such as dichloromethane, chloroform, acetone and dioxane, making them easily processable.

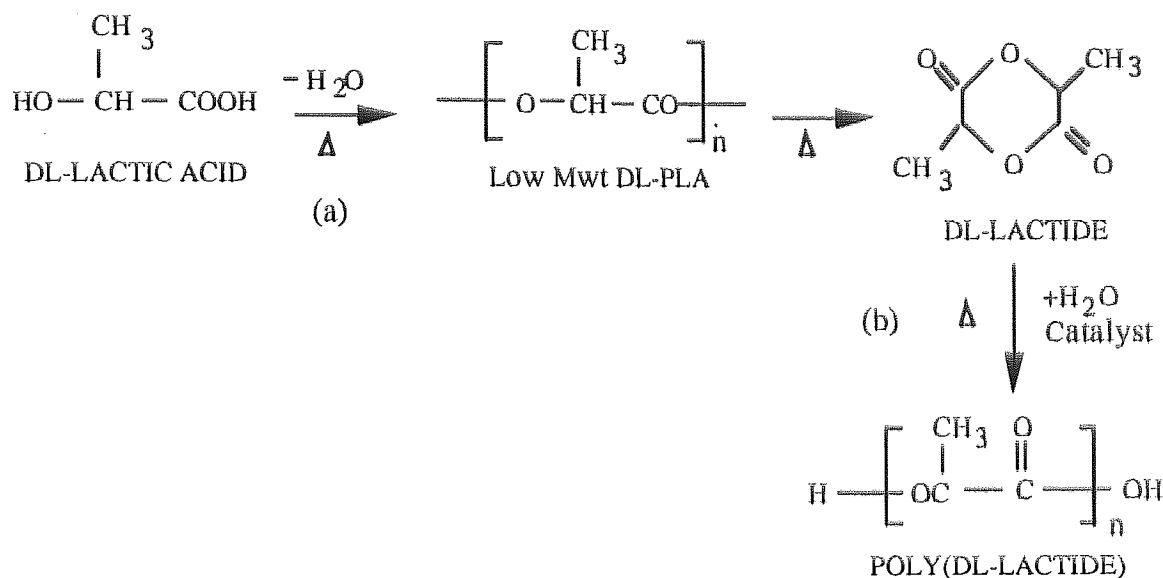


FIGURE 1.4 *Synthesis Of DL-Lactide Monomers (a) And Ring Opening Polymerization Of DL-Lactide (b) (adapted from Wise et al., 1979)*

PLA microparticles may be prepared by several different methods such as emulsion solvent evaporation, emulsion solvent extraction, interfacial phase deposition, spray coating, spray drying and the melting method. These processes are reviewed by Conti, *et al.*, (1992). Solvent evaporation is the most common preparation process for PLA microparticles (Benita *et al.*, 1984a; Cavalier, 1986; Jalil, 1990) (see Figure 1.5). The procedure consists of dissolving the polymer in a volatile organic solvent such as dichloromethane. The drug to be incorporated into the microparticles is then either dissolved or suspended in the same solution and the resulting mixture is emulsified in an aqueous phase that does not solubilize the polymer and contains an emulsifier. As the o/w emulsion is formed, the stirring rate is reduced and the solvent allowed to evaporate from the microdroplets formed, thereby giving solid drug-loaded microspheres. These may be isolated by filtration or centrifugation, washed, and dried under vacuum or freeze-dried.

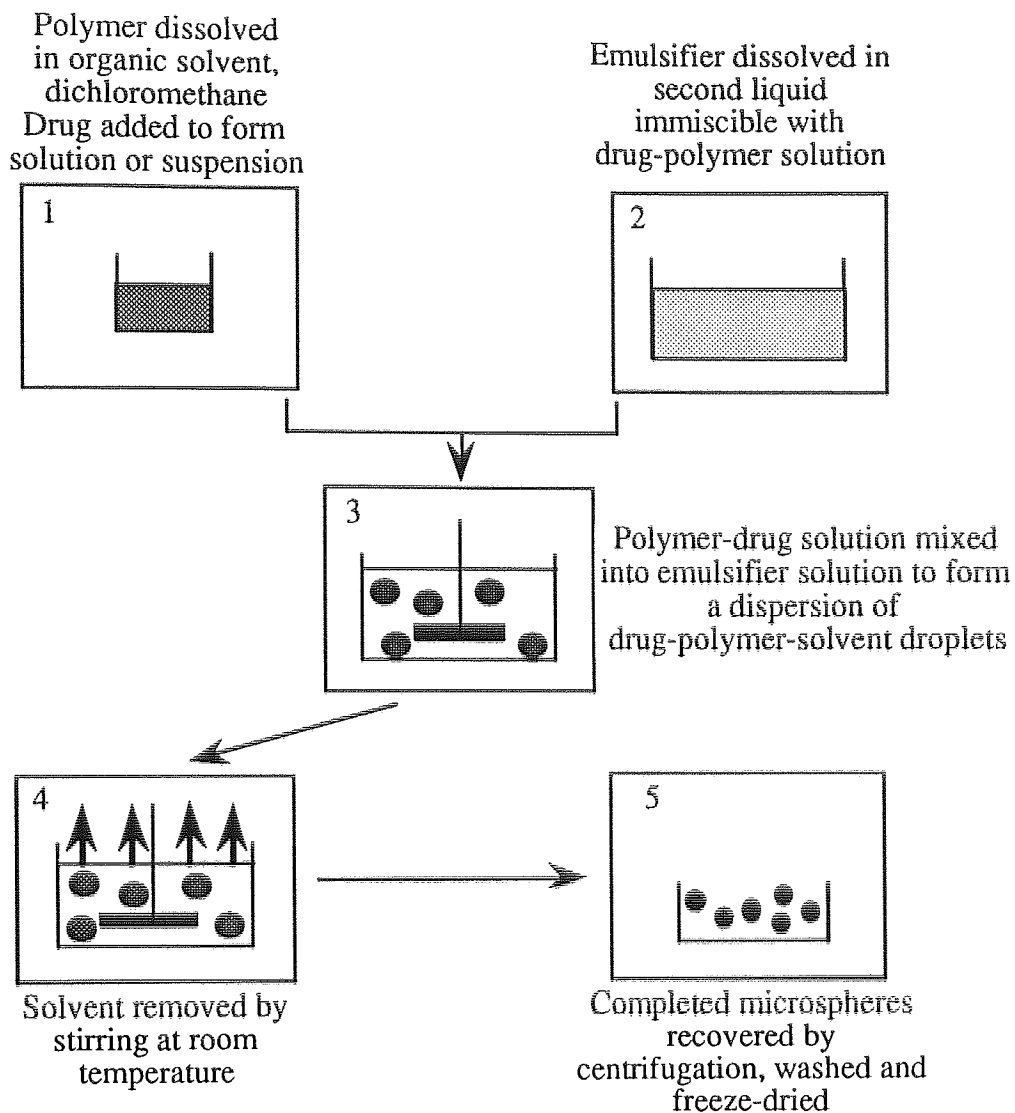


FIGURE 1.5 *Microsphere Formation By The Emulsification/Solvent Evaporation Technique (adapted from Watts et al., 1990)*

Many different types of drugs have been loaded into polylactide microparticles prepared by the solvent evaporation method, for both oral and parenteral drug delivery (reviewed by Watts *et al.*, 1990). In the latter system, there are two major potential applications; sustained release depots and targetable drug carriers. Parenteral sustained release depots have been used in medicine to provide sustained delivery of therapeutic agents, most commonly in the form of intramuscular injections: aqueous suspensions, oily solutions, oily suspensions and emulsions (Longer and Robinson, 1985). PLA has also been investigated as a potential drug delivery system for injectable implants. Beck *et al.*, (1979), proposed the use of progesterone-loaded DL-PLA microcapsules as an injectable, controlled release contraceptive system. Wakiyama *et al.*, (1981), evaluated DL-PLA microspheres as an injectable, sustained release delivery system for local anaesthetics. Prednisolone-loaded PLA microspheres have been tested for their

suitability as intra-articular implants (Smith & Hunneyball, 1986), and doxorubicin-loaded L-PLA microspheres have been investigated for intra-pleural administration to treat patients with pleuritis carcinomatosa (Ike *et al.*, 1990). Recently, much work has been focused on the potential use of PLA and poly(lactide-co-glycolide) microspheres as particulate carrier systems for vaccine delivery. Both injectable, sustained antigen release systems (O'Hagan *et al.*, 1991, Esparza & Kissel, 1992), and targetable antigen delivery systems to gut-associated lymphoid tissues (Eldridge *et al.*, 1990) and to nasal-associated lymphoid tissues (Almeida *et al.*, 1993) have been developed.

1.5 ANATOMICAL BARRIERS TO PARTICULATE DRUG DELIVERY

The major obstacles to effective site-specific drug delivery are the anatomical barriers which limit and govern the distribution of drugs. Two major barriers to the targeting of particles *via* the vascular compartment exist. Firstly, is the inability of the carrier to escape from the vascular system due to the endothelial cell barrier, and secondly, is the capture of particles in the vasculature by elements of the MPS.

1.5.1 THE ENDOTHELIAL BARRIER

In most tissues, the endothelial cells do not allow the escape of particulates that have dimensions from a few nanometers and/or larger. This is because the junctions between the cells are tight and the underlying basement membrane is also continuous in nature (Figure 1.6). In some tissues, particularly exocrine glands, the endothelial wall does contain small gaps or fenestrations (about 60 nm in size) but these are normally covered by a thin membrane. Even if a particle was able to escape through such a small fenestration, the basement membrane still presents an intact barrier. Only in a few specialized tissues, such as the spleen, the bone marrow and the liver, does the endothelial wall contain holes through which particles may escape, and where the underlying basement membrane is not intact. Here, there is an opportunity to reach the underlying parenchyma. A similar state of 'leakiness' in endothelial lining of blood capillaries can occur in areas of tissue inflammation and infection. The integrity of the endothelial barrier and underlying basement membrane are often disrupted during the inflammatory process, and the permeability thereof increased due to inflammatory oedema (Persson & Svensjö, 1985).

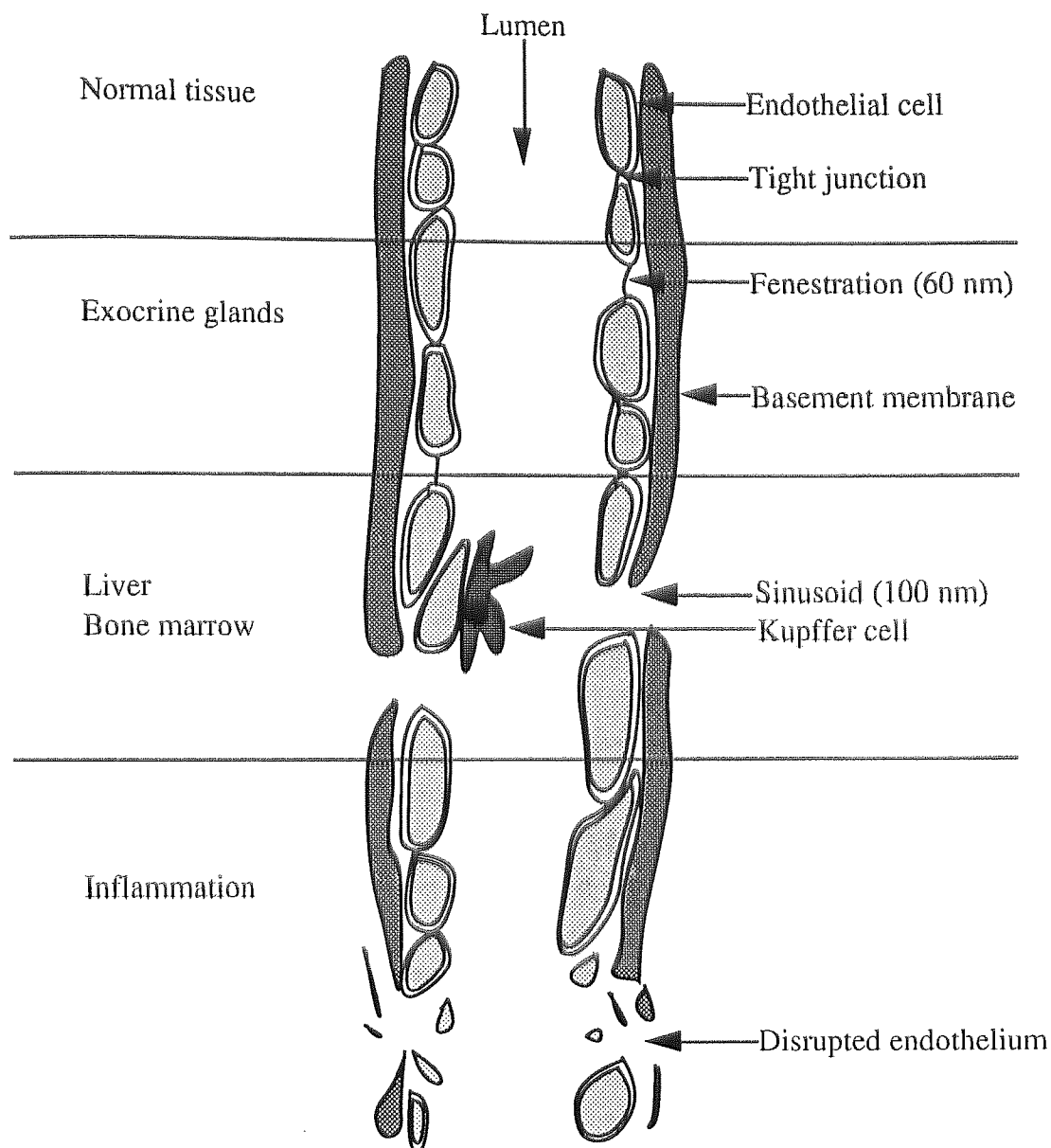


FIGURE 1.6 *Schematic Diagram Of The Endothelial Cell Barrier At Different Sites (adapted from Wright & Illum, 1992)*

1.5.2 THE MONONUCLEAR PHAGOCYTE SYSTEM

A second barrier to drug targeting is the interaction of particulates with cells of the MPS (reviewed by Poznansky & Juliano, 1984). The MPS is also known as the reticuloendothelial system (RES). The cell types comprising the MPS include circulating monocytes, the Kupffer cells of the liver sinusoids, splenic macrophages, mononuclear cells in the bone marrow, dendritic cells in the skin, and a variety of macrophage populations in tissues (Becker, 1988).

Cells of the MPS have a multitude of important biological functions (Becker, 1988), including the removal of protein aggregates derived from tissue destruction and repair, clearance of senescent erythrocytes and removal of invading bacteria, fungi and viruses. MPS cells are also highly efficient in removing foreign particles including microspheres, injected for either therapeutic or experimental purposes. Indeed, intravenously administered particulates may be removed from the vasculature within a few minutes (Illum & Davis, 1982). Although macrophage-like MPS cells are diffusely distributed in many tissues, the bulk of the MPS mediated clearance of injected particles occurs in the liver and the spleen.

Particles removed by phagocytosis are first attached to the macrophage, sometimes mediated by the adsorption of specific blood components termed opsonins. These include IgG, complement C3b and fibronectin. This attachment may involve a receptor mediated event, since the surface of the macrophage is known to have various receptors (that include those for immunoglobulins, complement and fibronectin), and non-specific receptors. The latter are able to mediate particle capture without the involvement of a specific blood component (De Duve *et al.*, 1974). Once the particle has been ingested it will find its way through various structures of the cell, including the phagosome and phagolysosome, to a final destination in the form of a digestion vacuole. These vacuoles in phagocytic cells contain a variety of enzymes and have the ability to digest a wide selection of natural and synthetic polymers (Duncan, 1986).

1.6 MICROSPHERE UPTAKE AFTER INTRAVENOUS ADMINISTRATION

The introduction of microparticulate carriers into the vasculature opens a range of opportunities to targets within the vasculature and to extravascular targets accessible *via* the capillary endothelium. The fate of a carrier administered into the vascular compartment will be determined by two main factors, particle size and particle surface characteristics.

If the particles are greater than about 7 μm , they will be removed by the filtering propensity of the lung. This passive uptake of particles represents one of the easiest approaches to targeting within the vascular compartment and it has been exploited much in radiodiagnostic imaging (Davis *et al.*, 1986), as well as in cancer chemotherapy (Willmott *et al.*, 1984). It is believed that the entrapment of drug-loaded microspheres in the lungs could allow higher concentrations of drug to reach the required sites.

Relatively small particles (less than about 7 μm) that are not removed by the lung will normally be removed by the cells of the MPS, if such particles are recognised as foreign (Bellanti, 1985). The MPS provides a rapid and efficient uptake of particles as described in Section 1.5.2, with nearly 100% of the injected particles having been reported to be removed from the circulation within a few minutes (Illum *et al.*, 1982). Thus, there is great opportunity for the passive targeting to macrophages. The clinical benefit that could be gained by targeting drugs to macrophages (reviewed by Davis & Illum, 1986), include the delivery of anti-infectives for the treatment of such diseases as leishmaniasis, brucellosis and candidiasis, lysosomal storage disease and macrophage antitumor activation for cancer chemotherapy (Tabata & Ikada, 1990).

In order to reach target sites outside the circulation, particles have to pass through the endothelial cell barrier. As discussed in Section 1.5.1, this is an effective barrier to particulates in normal tissue. However, extravasation of particulates may be aided by increased vascular permeability, for example, at sites of inflammation. Microspheres (Alpar *et al.*, 1989; Lewis *et al.*, 1992; Illum *et al.*, 1989) and liposomes (Love *et al.*, 1989) have been shown to be able to leak from the circulation at such sites.

1.6.1 STRATEGIES FOR AVOIDING THE MPS

If the cells of the MPS are not the desired target, it is obviously important to find a means with which to avoid this barrier. Two main methods to achieve this have been investigated; MPS suppression and alteration of the surface characteristics of the particulate drug carrier. These are discussed in further detail in the following sections.

1.6.1.1 MPS SUPPRESSION

An approach to preventing the uptake of particulates by cells of the MPS, especially the Kupffer cells, is to suppress the macrophages prior to microsphere administration with either a placebo particulate or macrophage suppressant agents like dextran sulphate 500 and methyl palmitate. Both dextran sulphate and multilamellar vesicles were investigated as agents for the suppression of the MPS in mice (Patel *et al.*, 1983). The injection of unlabelled vesicles prior to administration of labelled vesicles caused a 23% reduction in liver uptake with increased bone, lung and spleen uptake. Dextran sulphate was more effective with a decrease in liver uptake of 70%. In a similar study, Kao & Juliano, (1981), injected latex particles prior to injection of large liposomes. This approach had no effect on liver uptake, but spleen uptake was reduced by 45% and a higher lung uptake was observed. The effect of MPS suppression on the

distribution of subsequently administered particles was investigated by Illum and co-workers (Illum *et al.*, 1986). They found that liver suppression obtained using dextran sulphate or particles resulted in only 30% of the injected dose accumulating in the liver, the remaining being diverted to the lungs, spleen and bone marrow. Although successful in animal models, the clinical potential of this technique is doubtful since impairment of MPS functions can have serious consequences, especially in cancer patients (Poste & Kirsh, 1983).

1.6.1.2 ALTERATION OF PARTICLE SURFACE CHARACTERISTICS

As early as the mid 1960's, it was recognised that the surface charge on a particle has an effect on the biodistribution and clearance rate of parenterally administered particulates. Wilkins & Myers, (1966), altered the surface charge on polystyrene latex particles, using coatings of polylysyl gelatin and gum arabic, and found an alteration in half-life, with positively charged particles being removed less quickly than negatively charged ones. They also showed that after incubation in serum, particles with different surface characteristics acquire very similar electrophoretic mobilities, yet still showed different degrees of MPS uptake. Thus, interactions between the particles and the cells of the MPS must have been influenced by forces other than those of surface charge. Müller *et al.*, (1986), has since proposed that the surface hydrophobicity of a particle is related to its phagocytosis by cells of the MPS.

In vitro cell culture techniques have since been used to show the correlation between surface charge/hydrophobicity and phagocytosis. Microspheres with hydrophobic surfaces were found to be more readily phagocytosed by mouse peritoneal macrophages than those with hydrophilic surfaces (Tabata & Ikada, 1988), and underivatized polystyrene particles were found to stimulate neutrophils to a greater extent than particles bearing carboxyl, hydroxyl and amino groups (Orsini *et al.*, 1987). Therefore, it is likely that microspheres with hydrophilic surface properties are less likely to be sequestered to the MPS than microspheres with more hydrophobic properties. Many attempts have been made to alter surface hydrophobicity by coating microspheres with various hydrophilic agents. These include proteins (Ikada & Tabata, 1986; Torchilin *et al.*, 1980), polysaccharides (Takada *et al.*, 1984), and block copolymers (Davis *et al.*, 1993; Farr *et al.*, 1988; Illum & Davis, 1984, 1983; Leu *et al.*, 1984). The effect of altering the surface properties with these agents has been reviewed by Wright & Illum (1992).

Modification of the particle surface by the covalent attachment of hydrophilic groups such as polyethylene glycol has been reported (Artursson *et al.*, 1983, Davis *et al.*, 1993; Tomlinson, 1987), with particles modified in this way showing increased blood levels when compared to unmodified particles.

1.7 MICROSPHERE UPTAKE AFTER ORAL ADMINISTRATION

Peroral drug administration represents by far the most common and convenient route of drug delivery. However, in some cases this route is not feasible because of the occurrence of a number of problems. These include too short gastric residence times, lack of absorbability of drug, adverse drug side effects, and drug instability in the gastrointestinal tract (GIT). Biodegradable particulate drug delivery systems have been investigated over the past three decades as to their ability to overcome these problems. Knowledge of the mechanism of uptake of particulates across the GIT is a prerequisite to successful targeting to sites outside the GIT. However, the uptake and transport of particulates across the intestine is a controversial area.

For a long time, the wall of the mammalian GIT was assumed to be an impermeable barrier to the passage of inert particulates (O'Mullane *et al.*, 1987). In the early 1960's, however, the intestinal and subsequent hepatocellular uptake of small particles was demonstrated (Payne *et al.*, 1960; Sanders & Ashworth, 1961), and, since this time, many groups have investigated the uptake of particulates by the GIT. However, the mechanism and pathway of uptake of particulate matter across the GIT remains unclear. Several mechanisms of uptake are reported in the literature (reviewed by Kreuter, 1991; O'Hagan, 1990) and are summarised in Table 1.8. These proposed mechanisms of uptake fall into three main concepts: (1) transport across intact epithelial membranes, the transcellular route, possibly by endocytosis; (2) transport through endothelial tight junctions, the paracellular route; and (3) transport *via* the M-cells of Peyer's patches (PP). These routes are shown diagrammatically in Figure 1.7.

TABLE 1.8

Proposed Mechanisms Of Uptake Of Particulates Across The Gastrointestinal Tract (adapted from O'Hagan, 1990)

SITE/MECHANISM	SIZE RANGE	FATE AFTER UPTAKE	REFERENCE
Enterocyte/ Endocytosis	<220 nm	MPS uptake	Matsuno <i>et al.</i> , (1983)
Paracellular Transport	100-200 nm	Unknown	Aprahamian <i>et al.</i> , (1987)
Persorption	5-150 μ m	Blood and Excretory Fluids	Volkheimer (1993, 1977, 1968)
Peyer's Patches	20 nm-10 μ m	Peyer's Patches and MLN	Le Fèvre <i>et al.</i> , (1989)
Follicle-Associated Epithelium	<750 nm	MLN	Pappo and Ermak (1989)
Intestinal Macrophages	1 μ m	MLN	Wells <i>et al.</i> , (1988)

1.7.1 THE TRANSCELLULAR ROUTE

Absorption of macromolecules and particulates involving the process of endocytosis is complex and involves the formation of pinocytic vacuoles. The reported size range of the particles taken up in this fashion ranges from less than 20-40 nm (Barnett, 1959) up to 220 nm (Sanders & Ashworth, 1961). Intestinal absorption of macromolecules has been shown to occur in neonates and adults of various animal species (O'Hagan *et al.*, 1987). Similarly, endocytosis of particulates by enterocytes has also been reported (Matsuno *et al.*, 1983; Sanders & Ashworth, 1961). Accumulation of particulates in the mesenteric lymph nodes (MLN) before entering the systemic circulation, and subsequent phagocytosis by the Kupffer cells in the liver, appears to be the fate of the particles after uptake by endocytosis.

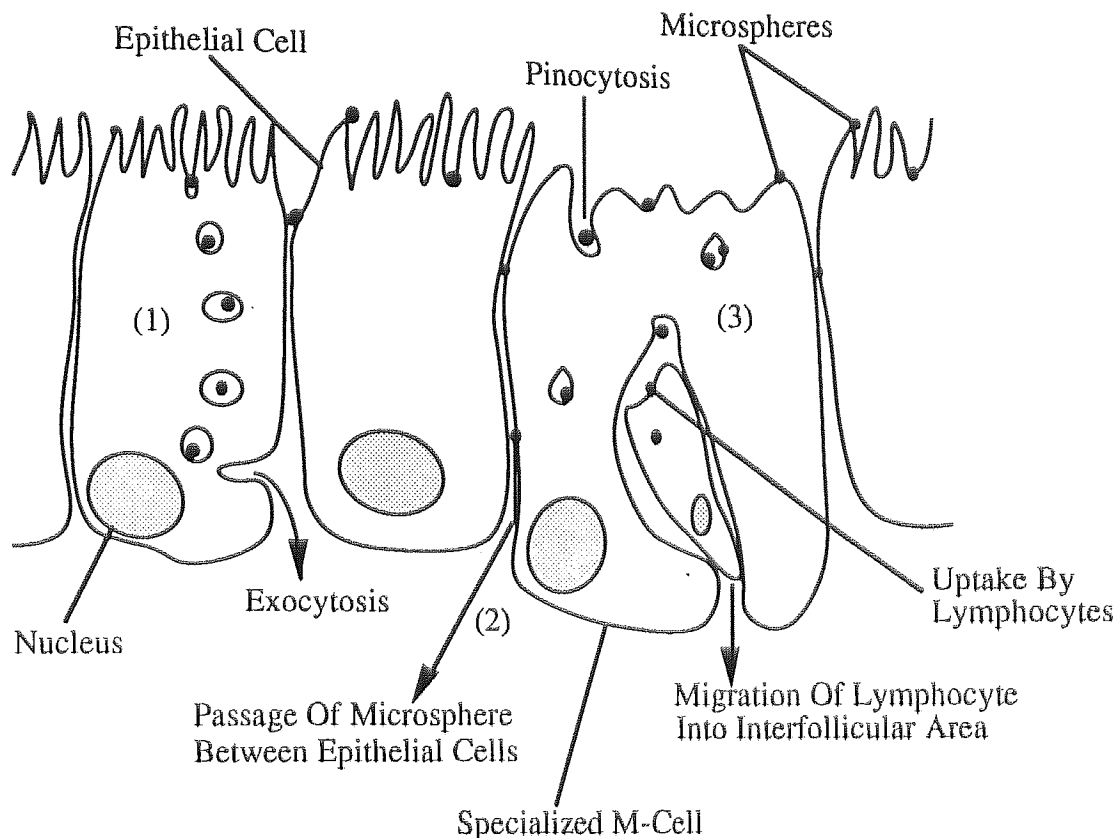


FIGURE 1.7 *Schematic Representation Of The Proposed Mechanisms Of Particulate Uptake Across The Gastrointestinal Tract. (1) The Transcellular Route, (2) The Paracellular Route, (3) Transport Via The M-Cells Overlying The Peyer's Patches (adapted from Florence & Jani, 1993)*

1.7.2 THE PARACELLULAR ROUTE

Paracellular transport of particulates involves their passage between the absorptive cells of the intestine, as opposed to intracellular transport, where the particulates pass through these cells. In normal circumstances, the tight junctions of the underlying gut epithelial cell membrane are thought to be an effective barrier against the absorption of all but the smallest molecules. However, in extensive studies, Volkheimer, (1977, 1968), investigated the uptake of a number of materials across the GIT including corn starch (3-25 μm), potato starch (5-10 μm), rice starch (3-10 μm), pollen (10-120 μm), diatoms (5-12 μm), poly(vinylchloride) particles (5-100 μm), cellulose particles, and plant cells. All of these particles were regularly demonstrable in blood and in the urine after peroral administration. Volkheimer speculated that particulate passage across the intestinal barrier resulted from the muscular activity of the muscularis mucosa, resulting in the 'kneading' of particulates between cells. Volkheimer proposed the term

'persorption' for this phenomenon and was the first to investigate the gastrointestinal uptake on a quantitative basis.

More recently, the paracellular transport of 100-200 nm nanocapsules have been reported (Aprahamian *et al.*, 1987). However, the nanocapsules were prepared in the presence of a surfactant, 0.5% Pluronic F68, which may have had a damaging effect on the gastric mucosa, thereby facilitating the passage of the nanocapsules across the GIT. Other treatments which have been reported to damage the epithelial cell membrane include aspirin (Meyer *et al.*, 1986), alcohol (Draper *et al.*, 1983), and hypertonic solutions (Wheeler *et al.*, 1978). The high, rapid uptake and appearance of particles in the blood, reported by Alpar *et al.*, (1989), and Eyles *et al.*, (1994), after peroral administration in relatively large dosage volumes of water, may be due to this damaging effect.

1.7.3 UPTAKE BY M-CELLS AND THE PEYER'S PATCHES

Membranous epithelial cells (M-cells) are specialized epithelial cells overlying lymphoid follicles in the gastrointestinal and respiratory tracts (Figure 1.8); a review by Wolf & Bye, (1984), has outlined their structure and nature. While solitary lymphoid nodules may occur along the entire intestine, they tend to be more numerous in the ileum, where they are grossly recognizable in aggregates as Peyer's patches (PP). PP are generally oval in shape and are usually located on the antimesenteric wall of the intestine. Their number changes with age, being maximal (around 300) at puberty and declining with age (Owen & Jones, 1974). M-cells have been ascribed an 'antigen-sampling' function and have been shown to be responsible for the uptake of bacteria, viruses and other pathogens across the mucosal barrier (reviewed in O'Hagan & Illum, 1990), thus allowing them access to cells of the immune system including lymphocytes, macrophages and plasma cells found in the PP.

The follicle-associated epithelium (FAE) that lines the lymphoid follicles of PP is composed of absorptive cells, goblet cells, M-cells and enteroendocrine cells. The FAE overlying PP contains a decreased number of goblet cells, compared with adjacent epithelium, resulting in a decrease in mucus secretion. This may make the M-cell's surface environment more conducive to antigen sampling and processing (Wolf & Bye, 1984). Similarly, M-cells may also therefore be more readily accessible for particulates.

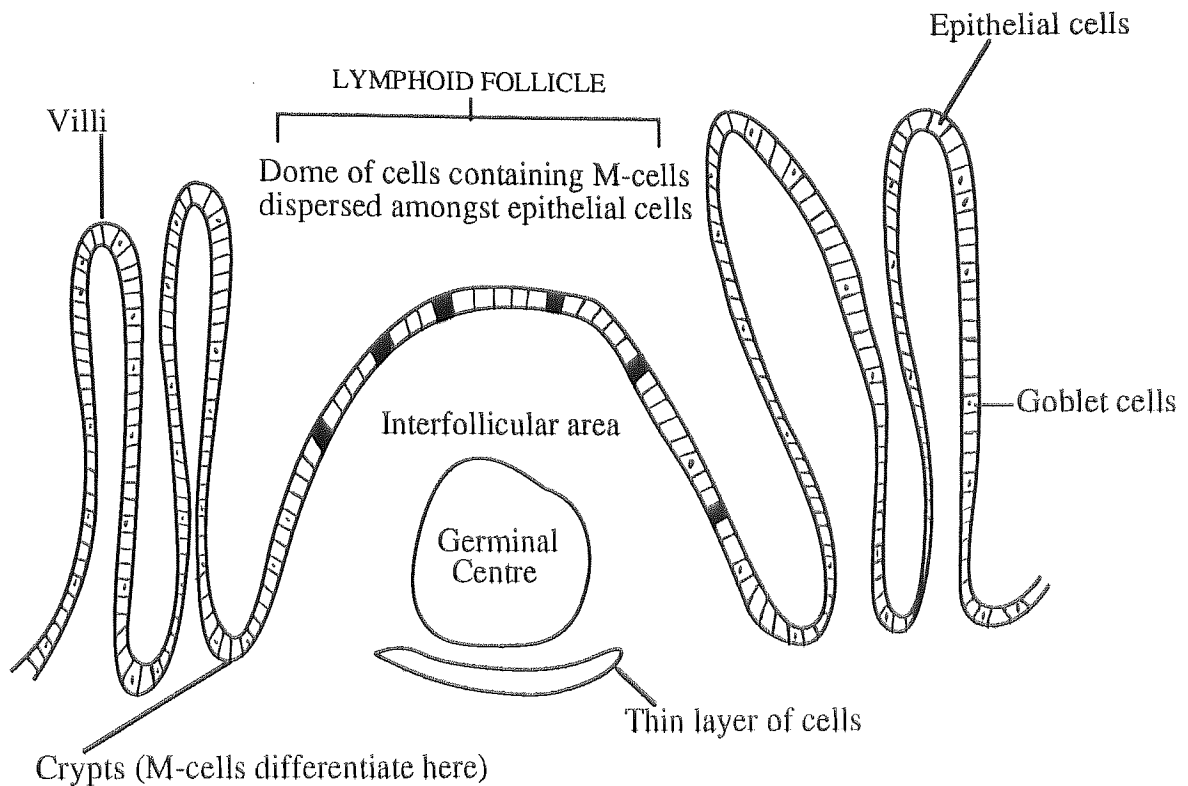


FIGURE 1.8 *Schematic Representation Of The Structure Of Intestinal Peyer's Patches Showing M-Cell Location In The Follicle-Associated Epithelium (adapted from O'Hagan, 1990)*

Several groups have implicated the M-cells of the FAE of the PP in the uptake and transport of particulates across the intestine. In a series of chronic feeding studies from 1970 onwards (reviewed in Le Fèvre & Joel 1984), Le Fèvre & Joel repeatedly demonstrated the accumulation of colloidal carbon (20-50 nm) and latex microspheres in the PP of animals that injected the particles orally. In a similar study (Jani *et al.*, 1990), the uptake of latex microparticles in the size range 50 nm to 3 μm , across the GIT was quantified by gel permeation chromatography. The uptake of latex particles and their subsequent passage into the MLN was demonstrated by Eldridge and co-workers (1990). Particles of greater than 5 μm left the PP and migrated through the MLN within phagocytic cells, while particles 5-10 μm stayed in the PP.

Isolated lymphoid follicles have also been shown to be capable of particulate uptake (Joel *et al.*, 1978). In a study by Wells and co-workers, (1988), in which fluorescent particles (1.09 and 0.89 μm) were implanted into isolated jejunal loops, particles were observed in phagocytic cells in MLN draining from the isolated intestinal segments. The segments used were devoid of PP but isolated lymphoid follicles were present. In

the same study, translocation of 6 μm fluorescent particles from ligated ileal loops were also reported.

1.7.4 TRANSLOCATION INTO THE BLOODSTREAM

From the existing body of experimental evidence there is no doubt that intestinally absorbed nanoparticles and even larger particles appear in the blood stream. One pathway of transportation into this body compartment is the described lymphatic uptake with subsequent entrance into the general circulation *via* the thoracic duct (Gilley *et al.*, 1988).

A second possible pathway is the direct delivery of the particles from the intestinal wall into the blood capillaries. Here, the particles have to cross two types of barrier after their transcellular or inter-epithelial uptake; the basement membrane and the wall of the capillaries (Aprahamian *et al.*, 1987). Komuro, (1985), using scanning and transmission electron microscopy techniques, reported the presence of fenestrations (0.5 μm - 5 μm in diameter) in the villous basal lamina of the small intestine, with about 1000 fenestrations on an average sized tongue-shaped villus. These surprisingly large fenestrations may explain the passage of particles through this barrier. The occurrence of channels formed by fenestral diaphragms of variable size, between less than 5 nm to 60 nm, in the endothelial epithelium (Bearer & Orci, 1985), may also permit nanoparticles to reach the capillaries (Aprahamian *et al.*, 1987).

1.8 ORIGINS AND SCOPE OF WORK

Drug delivery systems are designed to enhance the efficiency of therapeutic agents which may result in enhanced bioavailability, enhanced therapeutic index, and reduced side-effects. Conventional steroid therapy presents problems to patients in terms of both the unacceptable side-effects of systemic steroid therapy and the painful intra-articular injections of local steroid therapy.

Oral uptake of particulates across the GIT and into the systemic circulation, although remaining a controversial area, has been advocated by many groups (reviewed by Kreuter, 1991). Previous work by Alpar *et al.*, (1989), using latex (non-biodegradable) microspheres and inflammatory air-pouches in rats, demonstrated a rapid transport of microspheres from the GIT to the inflammatory air-pouches. As an extension of this work, Lewis *et al.*, (1992), manufactured biodegradable albumin microspheres and again reported their transportation into the circulation when orally

administered to rats. Furthermore, fluorescein isothiocyanate (FITC)-labelled albumin microspheres were also found to be present in the exudate fluids and tissues of carrageenan-induced inflamed rat hind-paws, although accurate quantification of the microspheres reaching this site of inflammation was not made. Hydrocortisone was incorporated into albumin microspheres by Lewis *et al.*, (1992), using a novel method of preparation, and steroid-loaded albumin microspheres were shown to be able to reduce inflammation of the rat hind paw more effectively than equivalent free steroid doses. This project, following the lines of investigation started by the forementioned workers (Alpar *et al.*, 1989; Lewis *et al.*, 1992), was designed to optimise the steroid-loading capacity and particle characteristics of albumin microspheres as an oral and intravenous drug delivery system, and to quantify the dose of particles reaching the sites of inflammation.

CHAPTER 2

PREPARATION AND CHARACTERIZATION OF ALBUMIN AND POLYLACTIC ACID MICROSPHERES

2. PREPARATION AND CHARACTERIZATION OF ALBUMIN AND POLYLACTIC ACID MICROSPHERES

2.1 INTRODUCTION

Over the past two decades, one of the most important objectives in drug therapy has been the selected delivery of therapeutic agents to specific target sites or organs in the body (Illum & Davis, 1982; Poste & Kirsh, 1983; Tomlinson, 1987). These systems have been designed to deliver therapeutic agents in the body in order to improve efficacy, safety, convenience and cost of therapy. As has been discussed in Section 1.3.2, there is a need for the development of greater site-specificity for the currently available drugs used in the therapy of RA. The treatment of this clinically heterogenic disease is complicated by adverse reactions and unwanted side-effects of the drugs available, and, along with little clinical improvement for patients, poor patient compliancy. Scientists have investigated the possible use of injectable, sustained drug delivery systems for intra-articular use (Foong & Green, 1993, 1988; Ratcliffe *et al.*, 1987; 1984), thereby reducing the number of i.a. injections necessary to sustain effective drug levels within the synovial joint. Also, investigations have been undertaken to develop targetable drug delivery systems to the site of inflammation for parenteral and non-parenteral administration (Burgess & Davis, 1988; Burgess *et al.*, 1987; Illum *et al.*, 1989; Lewis *et al.*, 1992). As with all parenterally and non-parenterally administered drug delivery systems, the anatomical barriers which particulates must surmount to reach specific sites within the body are a major obstacle in targeted drug delivery (described in Section 1.5). As has been discussed in Sections 1.6 and 1.7, particle size and surface charge are important characteristics in the biodistribution patterns of particulates *in vivo*.

Previous work by Illum *et al.*, (1989), has shown the accumulation of intravenously injected latex particles (80 nm mean diameter), labelled with iodine-131 and coated with Poloxamine 908, at the site of carrageenan-induced chronic inflammation in rabbits. Alpar *et al.*, (1989), showed the accumulation of FITC-labelled latex particles (1.1 μm mean diameter) in inflammatory air-pouches in the rat after administration directly into the heart. The same group also showed the accumulation of FITC-labelled latex particles (1.1 μm mean diameter) in inflammatory air-pouches in the rat, after oral administration of the particles by gavage. Lewis *et al.*, (1992), reported the presence of FITC-labelled albumin microspheres (mean diameter 3.1 μm) in inflamed tissues and exudates in carrageenan-induced hind-paw inflammation in the rat, after oral dosing by gavage.

In light of these findings, it would appear that anatomical barriers to site-specific drug targeting may be overcome, with particulates being able to accumulate at inflammatory sites. The aim of this study was to prepare albumin microspheres of suitable size and surface characteristics, which would be able to reach sites of tissue inflammation after oral and intravenous administration. For *in vivo* tissue distribution studies, it was necessary to radiolabel albumin microspheres so that quantitative measurements of the dose of administered microspheres reaching the site of inflammation could be determined. Both albumin and PLA microspheres were prepared for future steroid incorporation studies.

2.2 MATERIALS

Poly (L-lactide), 2,000 Da molecular weight (Mwt) and 57,000 Da Mwt, manufactured by Boehringer Ingelheim, were purchased from Alfa Chemicals Ltd, Preston, England. PVA (10,000 Da Mwt, 88% hydrolysed) was purchased from BDH Chemicals Ltd., Poole, UK. [¹²⁵I]-Bovine serum albumin (BSA) was purchased from NEN Research Products, Dupont UK Ltd., Stevenage, UK. All other chemicals were of Analar or reagent grade and were purchased from appropriate suppliers as listed in Appendix I. Distilled water was used for all solutions and suspensions unless otherwise stated.

2.3 INSTRUMENTATION

An Omni-Mixer (OCI Instruments, UK) having a four-bladed paddle and metal bucket attachment, and a Silverson Homogenizer Model STL2 (Silverson Machines Ltd., Chesham, UK) were used for emulsification purposes. An electric motor (Citenco Motors, England) was employed to rotate a glass paddle, the rate of which was measured with a tachometer. A Sarstedt LCI centrifuge and a Beckman J2-21 centrifuge (Beckman Instruments Ltd, Buckinghamshire, England) were used for centrifugation processes. An Edwards Modulyo freeze-drier (BOC Ltd, Sussex, England) was employed for freeze-drying purposes. The following Sartorius electronic balances (Sartorius GMBH, Göttingen, Germany) were employed for accurate weighing purposes; an analytical A202S (4 decimal places) and a research R202D (5 decimal places). A Kerry Laboratory Sonicator was used to aid suspension of microspheres where necessary. A flask shaker (SFI, Stuart Scientific Company Ltd., UK) was employed for gentle mixing purposes.

For scanning electron microscopy (SEM) purposes an Emscope SC500 Sputter Coater and a Cambridge Stereoscan 90 SEM (Cambridge Instruments, England) were employed. Photomicrographs were taken with a Nikon F301 35 mm camera.

Zeta potential measurements were analysed by laser Doppler anemometry using a Zetameter (Malvern ZetaSizer 4, Malvern Instruments Ltd., Malvern, England) provided with a digital correlator (Series 7032 Multi-8, Malvern Instruments Ltd.).

2.4 METHODS

2.4.1 ALBUMIN MICROSPHERE PREPARATION

Several methods have been reported in the literature for the manufacture of albumin microspheres (reviewed by Gupta & Hung, 1989a). Routine preparation of these particles utilizes an aqueous solution of protein and a suitable vegetable oil to form a w/o emulsion. The emulsion is stabilized after transferring to an oil medium, so as to harden and subsequently isolate the protein microspheres. Stabilization of the emulsion maintains the rigidity of the particles and minimizes their coalescence. Two methods are predominately employed for the stabilization of albumin microspheres, namely thermal-denaturation and chemical cross-linking (see Figure 1.3).

Modifications of reported methods (Gallo *et al.*, 1984; Lewis *et al.*, 1992; Scheffel *et al.*, 1972) were used to develop a suitable technique for thermal-denatured albumin microspheres (see Table 2.1, D). Similarly, modifications were made to reported methods (Ratcliffe *et al.*, 1984; Yapel, 1979) for chemically cross-linked albumin microsphere preparation (Table 2.2, G). These methods, described in Sections 2.4.1.1 and 2.4.1.2, were then adopted for all ensuing microsphere preparations.

2.4.1.1 THERMAL-DENATURED ALBUMIN MICROSPHERE PREPARATION

Thermal-denatured albumin microspheres were prepared by the following method. 500 μ l aqueous BSA solution (25% w/v) was added to 30 ml olive oil and homogenized (\geq 15,000 rpm) for 2 min with an Omni-Mixer. The w/o emulsion formed was added drop-wise (40 ± 10 drops min^{-1}) into a round-bottomed flask containing 100 ml olive oil at $125 \pm 5^\circ\text{C}$, being stirred at 600 rpm using a two-paddled glass rod and electric motor. Heating and stirring was continued for 1 hr followed by cooling to $20 \pm 5^\circ\text{C}$. 60 ml n-heptane was added and the microspheres harvested by centrifugation (2,000 rpm, 15 min). The prepared microspheres were washed by centrifugation (2,000 rpm, 15 min).

three times in 60 ml aliquots of diethyl ether, before being freeze-dried overnight. The microspheres were stored at room temperature in a desiccator.

TABLE 2.1 *Modifications To Thermal-Denatured Albumin Microsphere Preparation Methods Cited In The Literature*

METHOD	AQUEOUS PHASE (ml)	ALBUMIN CONC. (% w/v)	OIL PHASE FOR EMULSIFICATION	EMULSIFICATION STEP	TIME (hr)	TEMPERATURE (°C)	STIRRING RATE OF OIL PHASE† (rpm)
Scheffel <i>et al.</i> , (1972)	1	25	100 ml Cotton-seed oil	Hand-operated homogenizer	0.17	175-185	1,700
Gallo <i>et al.</i> , (1984)	0.5	50	30 ml Cotton-seed oil	Ultrasonication 125 W, 2 min	0.17	125±5	1,500
Lewis <i>et al.</i> , (1992)	0.5	50	30 ml Olive oil	Homogenization 1 min 14,000 rpm	0.17	125±5	Not reported
A	1	25	30 ml Olive oil	Homogenization 2 min 15,000 rpm	1	125±5	600
B	0.5	50	30 ml Olive oil	Homogenization 2 min 15,000 rpm	1	125±5	600
C	0.5	25	30 ml Olive oil	Homogenization 2 min 15,000 rpm	1	125±5	600
D	0.5	25	60 ml Olive oil	Homogenization 4 min 15,000 rpm	1	125±5	600

† 100 ml of oil was employed at the stated temperature and stirring rate for the stated length of time in order to thermally denature the albumin microspheres

TABLE 2.2 *Modifications To Chemically Cross-Linked Albumin Microsphere Preparation Methods Cited In The Literature*

METHOD	AQUEOUS PHASE (ml)	ALBUMIN CONC. (% w/v)	OIL PHASE	EMULSIFICATION STEP	CROSS-LINKING AGENT†	TIME (hr)	STIRRING RATE (rpm)
Yapel (1979)	2.0	50	500 ml Cotton-seed oil	Injected into oil phase stirring at 1200 rpm	Glutaraldehyde (25% aq) 0.4% v/v	4	1,200
Ratcliffe <i>et al.</i> , (1984)	0.2	12.5	25 ml Olive oil	Heidolph Type RR1 Mixer 5 min	Glutaraldehyde (25% aq) 0.1 ml	0.25	rapidly stirred
E	0.5	50	30 ml Olive oil	Homogenization 2 min 15,000 rpm	Glutaraldehyde (25% aq) 0.1 ml	0.5	200
F	0.5	50	30 ml Olive oil	Homogenization 2 min 15,000 rpm	Glutaraldehyde (25% aq) 0.1 ml	1	600
G	0.5	25	30 ml Olive oil	Homogenization 2 min 15,000 rpm	Glutaraldehyde (25% aq) 0.1 ml	1	600

†Chemical stabilization was carried out at room temperature in all methods

2.4.1.2 CHEMICALLY CROSS-LINKED ALBUMIN MICROSPHERE PREPARATION

Chemically cross-linked albumin microspheres were prepared using glutaraldehyde (25% aqueous solution) as the chemical stabilizing agent. The w/o emulsion was formed as described for thermal-denatured albumin microspheres. To initiate the cross-linking process, 100 μ l glutaraldehyde (25% aqueous solution) was added to the emulsion being stirred at 600 rpm with a paddled glass rod. Stirring was continued for 1 hr to complete the stabilization process, after which 60 ml n-heptane was added and the microspheres harvested by centrifugation (2,000 rpm, 15 min). The prepared microspheres were washed by centrifugation (2,000 rpm, 15 min) three times in 60 ml aliquots of diethyl ether before being freeze-dried overnight. The microspheres were stored at room temperature in a desiccator.

2.4.1.3 RADIOLABELLED ALBUMIN MICROSPHERE PREPARATION

In order to trace albumin microspheres *in vivo* for tissue distribution experiments, microspheres were prepared with radioiodinated BSA. Previous studies by Ratcliffe *et al.*, (1987), prepared their own radiolabelled serum albumin with [¹³¹I], and utilized this successfully in their microsphere manufacturing procedure. In these studies, [¹²⁵I]-BSA was supplied in 1 ml sterile 0.05 M sodium phosphate solution, pH 7.5 (57.3 MBq ml⁻¹). This was pipetted into 10 equal aliquots and further diluted to give a working solution of 5 MBq ml⁻¹. A 31.25% w/v BSA solution was prepared and 400 µl of this solution added to 100 µl [¹²⁵I]-BSA working solution. 500 µl of this 25% w/v [¹²⁵I]-BSA solution was used to prepare radiolabelled albumin microspheres by the chemical cross-linking method outlined in Section 2.4.1.2 with a single modification. This involved two further washing steps in diethyl ether to remove any remaining uncrosslinked [¹²⁵I]-BSA from the microsphere preparation. All radiolabelled microsphere preparations procedures were carried out using appropriate [¹²⁵I] handling and disposal practices, and safety shielding equipment.

In order to quantify the amount of uncrosslinked [¹²⁵I]-BSA remaining in the final microsphere preparations, the radioactivity of small samples of each batch of microspheres were counted, the samples were then suspended in 1 ml PBS and incubated in a shaking water bath (Tecam 5B-16, Techne Ltd., Cambridge, UK) at 37°C for 4 hr and 24 hr. After each time period the microspheres were vortexed thoroughly for 2 min, harvested by centrifugation and the supernatants assessed for radioactivity.

2.4.2 PLA MICROSPHERE PREPARATION

PLA is used in the manufacture of microspheres predominately prepared by the solvent evaporation method described by Beck *et al.*, (1979) (see Figure 1.5). The polymer is dissolved in an organic solvent (*eg.*, dichloromethane) and this is added to a vigorously stirred aqueous phase containing an emulsifier (*eg.*, polyvinyl alcohol, [PVP]) to help stabilize the dispersed polymer droplets. An o/w emulsion is thus formed. Evaporation of the organic solvent from the droplets results in the hardening of the polymer microspheres. Drugs may be incorporated into the microspheres by dissolving or suspending the drug of interest in the organic phase prior to use.

Modifications of reported methods (Benita *et al.*, 1984a; Wichert & Rohdewald, 1990), based on the original method of Beck *et al.*, (1979), were used to develop a suitable

technique for the preparation of PLA microspheres by the solvent evaporation method (see Table 2.3).

TABLE 2.3 *Modifications To Some PLA Microsphere Preparation Methods Reported In The Literature Based On The Original Solvent Evaporation Method Of Beck et al., (1979)*

METHOD	AQUEOUS PHASE (ml)	ORGANIC PHASE (CH ₂ Cl ₂) (ml)	PLA (g)	EMULSIFIER	TEMPERATURE (°C)	STIRRING RATE (rpm)	TIME (hr)
Benita <i>et al.</i> , (1984a)	250	20	D,L-PLA (Mwt 61,000) 0.927	PVA (1.5%)	22	450	7-17
Wichert & Rohdewald (1990)	40	1	D,L-PLA (Mwt 16,000) 0.12	Gelatin B (0.5%)	room temp.	≈10,000	0.5
H	75	5	L-PLA (Mwt 2,000) 0.25	PVA (1.5%)	4	3,000	17
I	75	5	L-PLA (Mwt 2,000) 0.25	PVA (1.5%)	4	≥10,000	17
J	75	5	L-PLA (Mwt 57,000) 0.25	PVA (1.5%)	4	3,000	17
K	75	5	L-PLA (Mwt 57,000) 0.25	PVA (1.5%)	4	≥10,000	17

Method I was used for all ensuing PLA microsphere preparations. The internal, organic phase consisted of 250 mg poly (L-lactide), 2,000 Da Mwt, being dissolved in 5 ml of dichloromethane. The external, aqueous phase consisted of a 75 ml solution of 1.5% PVA (Mwt 10,000 Da, 88% hydrolysed) in distilled water. Both the organic and aqueous phases were cooled in an ice bath for 1 hr prior to microsphere preparation. This results in an increase in viscosity of the solutions which could act as an additional factor for stabilization of the final emulsion. An o/w emulsion was formed by the dropwise addition of the organic phase to the aqueous solution of PVA, being stirred at a rate of $\geq 10,000$ rpm with a Silverson homogenizer. After stirring for a further 5 min, the mixture was magnetically stirred overnight, allowing the evaporation of the organic solvent. The low temperature of the preparation may slow the evaporation of the organic solvent, contributing to the formation of regular shaped, spherical microspheres with a smooth surface. The particles were harvested by centrifugation (10,000 rpm, 30 min), washed three times in distilled water by centrifugation (10,000 rpm, 15 min), and freeze-dried overnight. The microspheres were stored desiccated at room temperature.

2.4.3 PARTICLE SIZE ANALYSIS

Particle size was manually determined by SEM examination. A small amount of freeze-dried microspheres were spread onto a SEM stub covered with double-sided selotape. An anti-electrostatic gun was used to remove any electrical charge from the stub before a 20 nm gold film was deposited onto the sample surface in a sputter coater. The microspheres were viewed by random scanning of the stub in the SEM. Photomicrographs were taken of monolayers of microspheres and these were used for microsphere diameter measurements ($n \geq 100$). The particle size was statistically analysed in order to assess the size distribution and to determine the geometric mean and geometric standard deviation (σ_g), as described by Martin *et al.*, (1983).

2.4.4 ADSORPTION OF POLOXAMINE 908 ONTO ALBUMIN MICROSPHERES

The major obstacle for drug delivery with intravenous microspheres is the uptake of the particles by cells of the MPS (discussed in greater detail in Section 1.6). Particulate uptake by cells of the MPS has been shown to be reduced by the adsorption of ethoxylated block copolymers (poloxamer) onto the surface of 60 nm latex microspheres (Illum & Davis, 1984; 1983). The adsorption (coating) protected the particles against cellular uptake enabling them to circulate in the blood (Davis *et al.*, 1986; Illum *et al.*, 1987). The adsorption of poloxamer polymers and Poloxamine 908 onto the surface of biodegradable microspheres has been described in detail by Müller

& Wallis, (1993), with adsorption onto the particle surface been confirmed by measuring the surface layer thickness and surface potential of the particles by photon correlation spectroscopy and laser Doppler anemometry respectively.

Lewis *et al.*, (1992), investigated the tissue distribution kinetics of orally administered FITC-albumin microspheres in a 1% solution of Poloxamine 908. Results showed that this did not alter the rate or number of microspheres transported across the GIT within the first hour of dosing, but allowed particles to be detected in the blood 24 hr after dosing, when compared to microspheres administered in water. No further studies were made to characterize the adsorption of Poloxamine 908 onto the albumin microsphere surface. Since the tissue distribution of albumin microspheres in this study was aimed at intravenous as well as oral administration, a preliminary study to investigate whether Poloxamine 908 may be adsorbed onto the surface of albumin microspheres was undertaken, with possible coating being determined by zeta potential measurements.

The method used for the adsorption of Poloxamine 908 onto albumin microspheres was adapted from the methods of Müller & Wallis (1993), and Illum *et al.*, (1986), who used 0.2% w/v and 2.0% w/v solutions of Poloxamine 908 and Poloxamer 188 respectively, with overnight incubation at room temperature.

Chemically cross-linked albumin microspheres were added to 0.2% w/v and 2.0% w/v Poloxamine 908 solutions and incubated for 30 min at room temperature with gentle agitation using a flask shaker. This short time of incubation was used to prevent the possible swelling of albumin microspheres over a 24 hr period, as particle size has been reported to alter zeta potential measurements (Tabata & Ikada, 1988). After incubation, the microspheres were harvested by centrifugation (2,000 rpm, 5 min), and resuspended in quarter strength PBS (Appendix II). Zeta potential measurements were taken immediately as described in Section 2.4.5.

2.4.5 ZETA POTENTIAL MEASUREMENT OF ALBUMIN MICROSPHERES

The surface charge carried by microspheres may be determined by measuring the particle mobility in an electric field, then, by using the equations of Smoluchowski, Henry or Debye-Hückel (Müller *et al.*, 1986), the zeta potential (ζ) can be calculated. The electrophoretic mobility (μ_E) is defined as the particle's velocity under electric field and usually expressed in relation to the particle velocity (v) and the applied field strength (E), as in the equation,

$$\mu_E = v/E$$

where v is measured in ms^{-1} , and E in Vm^{-1} , so that μ_E has the dimensions $\text{m}^2\text{s}^{-1}\text{V}^{-1}$ (Kayes, 1988). The equation used for converting the electrophoretic mobility into zeta potential depends upon the values of the Debye-Hückel parameter (k), which depends upon the electrolyte concentration, and the particle radius (a). At large values of ka the Smoluchowski equation can be applied. In the present work, given the conditions of measurement (aqueous medium, moderate electrolyte concentration, and an average particle radius of approximately $2.5 \mu\text{m}$), this was the equation used:

$$\zeta = \mu_E \{4 \pi \eta / \epsilon\}$$

where η is the viscosity of the medium (in Pascals) and ϵ is the respective dielectric constant. The value of ζ is measured in mV. It follows from this equation that the electrophoretic mobility of a non-conducting particle for which ka is large at all points on the surface, should be independent of its size and shape, provided the zeta potential is constant (Shaw, 1980).

Microsphere preparations were dispersed by a 1 min sonication in a quarter strength PBS solution, and analysed by laser Doppler anemometry using a zetameter. Determinations were undertaken using the parameters shown in Table 2.4.

TABLE 2.4 *Parameters Used In Zeta Potential Measurements*

PARAMETER	VALUE
Cell Type	AZ104
Cell Voltage	86.25 ± 0.85 V
Current	8.26 ± 0.30 mA
Conductivity	4.79 ± 0.17 mS
Viscosity	0.93 ± 0.004
Temperature	$23 \pm 0.5^\circ\text{C}$
Dielectric Constant	79.0
$f(ka)$	1.5 (Smoluchowski)

2.5 RESULTS AND DISCUSSION

2.5.1 ALBUMIN MICROSPHERE PREPARATION

There still remains much controversy as to the size of particulates which may be transported across the GIT and pass into the systemic circulation after oral administration. Previous reports of the size of particulates being able to reach the systemic circulation range from 20 nm up to 150 μm (Table 1.8). Volkheimer (1993), reported the persorption of particulates with diameters ranging from 3-150 μm . Eldridge *et al.*, (1990), reported particles should be less than 10 μm , preferably $\leq 5 \mu\text{m}$, and these results contradicted those of Jani *et al.*, (1990), who reported the uptake of particulates increased as the size decreased from 3 μm to 50 nm. The latter two groups both concentrated on using commercially available preparations of non-biodegradable microspheres. More recently, Lewis *et al.*, (1992), reported microspheres with a mean diameter of about 3.1 μm were transported across the GIT and found in the systemic circulation after oral dosing. These investigations, similar to the ones to be undertaken in these studies, were made with biodegradable, albumin microspheres. As opinions of particulate size differ, and the type of microspheres used in these studies also differ, the aim of this study was to prepare biodegradable microspheres with mean particle diameters $< 3 \mu\text{m}$.

Both thermally-denatured and chemically cross-linked albumin microspheres were prepared, with adaptations of methods cited in the literature, to achieve a mean particle size $< 3 \mu\text{m}$. Factors affecting size and size distribution of albumin microspheres have been investigated by many groups, based on factorially designed experiments (Torrado *et al.*, 1989, Gupta *et al.*, 1988), and are summarized in Table 2.5.

Method C was chosen for routine manufacture of thermally-denatured albumin microspheres, with this method being reproducible in terms of mean particle diameter under the same experimental conditions ($n \geq 3$). All thermal-denatured albumin microsphere batches gave rise to cream, free flowing powders without particle aggregation and coalescence. When observed under SEM, the microspheres had a smooth surface and were spherical (Plate 2.1). Particle diameters were measured manually, with ≥ 100 microspheres being measured for each batch. Each batch data was represented in the form of a frequency size distribution curve, which repeatedly gave rise to slightly skewed size distribution curves, an example of which is shown in Figure 2.1. When the data shown in Figure 2 was plotted as frequency versus the logarithm of the particle diameter, a bell-shaped curve was obtained *i.e.*, a log-normal

distribution. From the log-normal distribution the geometric mean diameter and geometric standard deviation was determined as described by Martin *et al.*, 1983.

TABLE 2.5 *Factors Affecting Size And Size Distribution Of Albumin Microspheres (from Gupta & Hung, 1989a)*

FACTOR
Drug concentration
Protein concentration
Surfactant concentration
Degree of homogenization during emulsification
Degree of carrier stabilization
Degree of agitation during carrier stabilization
Design of apparatus
Type of oil

Table 2.6 shows some of the mean particle sizes, measured by SEM, achieved for thermal-denatured albumin microspheres after modifying the methods of Scheffel *et al.*, (1972), Gallo *et al.*, (1984), and Lewis *et al.*, (1992). The first preparation method, A, produced microspheres with a mean diameter too large for the purposes of the present work. However, methods B, C, and D all produced microspheres with mean diameters <3 μm . Method D produced microspheres with the smallest mean diameter and the most homogeneous microsphere population. It is known that the degree of ultrasonication or homogenization, as well as the length of homogenization time, may affect the size and size distribution of albumin microspheres. An increase in the level of homogenization has been shown to result in the production of smaller microspheres with narrower size distributions (Gallo *et al.*, 1984; Longo *et al.*, 1982). Problems were encountered, however, with overheating of the homogenizer during the emulsification step, and this method of microsphere preparation was not, therefore, routinely used. Method B showed a slightly higher mean diameter than method C. The difference in these two methods was in the albumin concentration, with that in method B being twice that in C. An increase in protein concentration leads to its increased viscosity in water. Hence its subdivision into smaller particles becomes relatively difficult (Ishizaka *et al.*, 1981), and larger droplets are formed, consequently leading to larger mean particle diameters.

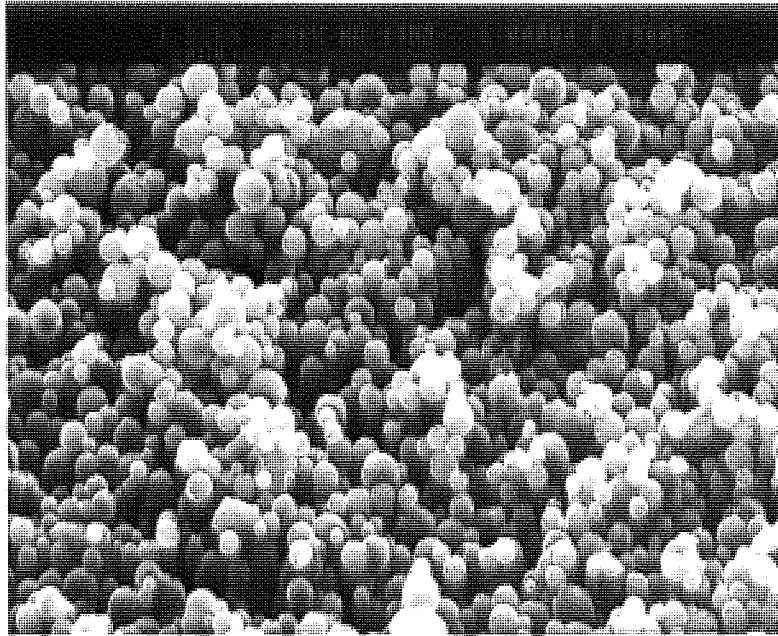


PLATE 2.1 *Scanning Electron Micrograph Of Thermal-Denatured Albumin Microspheres*

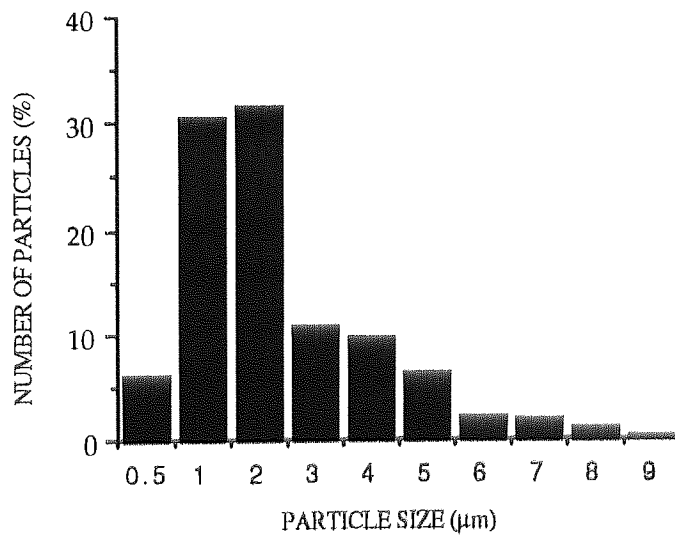


FIGURE 2.1 *Particle Size Distribution Of Thermal-Denatured Albumin Microspheres Prepared By Method C. Geometric Mean Diameter = 2.47 μm; $\sigma_g = 1.84 \mu\text{m}$*

TABLE 2.6 *Mean Particle Size Obtained With Some Variations Of The Methods Cited In The Literature Used To Prepare Thermal-Denatured Albumin Microspheres*

PREPARATION METHOD	MEAN SIZE (μm)
Scheffel <i>et al.</i> , (1972)	0.4 - 0.6*
Gallo <i>et al.</i> , (1984)	$0.44 \pm 0.30^\dagger$
Lewis <i>et al.</i> , (1992)	$3.10 \pm 1.70^+$
A	$4.35 \pm 3.52^+$
B	$2.69 \pm 1.98^+$
C	$2.47 \pm 1.84^+$
D	$2.20 \pm 1.12^+$

*Particles examined by SEM. Most frequently occurring diameter reported to vary between this size.

†Geometric mean particle size diameter and standard deviation, manually measured from SEM photomicrographs (n \approx 500)

+Geometric mean particle size diameter and standard deviation, manually measured from SEM photomicrographs (n \geq 100)

The chemical cross-linking method of albumin microsphere preparation has been used as an alternative to thermal-denaturing when incorporating heat labile drugs. In this study, glutaraldehyde cross-linking was investigated as a means of preparing [^{125}I]-labelled microspheres for *in vivo* tissue distribution experiments.

Method G was chosen for routine manufacture of glutaraldehyde cross-linked albumin microspheres, with this method being reproducible in terms of mean particle diameter under the same experimental conditions (n \geq 3). The microspheres were produced in a tan, free flowing powder without particle aggregation and coalescence. When observed under SEM, the microspheres had a smooth surface and were spherical (Plate 2.1). Again, each batch data was represented in the form of a frequency size distribution curve, which repeatedly gave rise to slightly skewed size distribution curves, an example of which is shown in Figure 2.2, along with the calculated geometric mean and σ_g for this microsphere batch.

Glutaraldehyde cross-linked albumin microspheres were prepared based on the methods of Yapel (1979) and Ratcliffe *et al.*, (1984). Table 2.7 shows some of the

mean particle sizes, measured by SEM, achieved. Method E gave rise to microspheres with a mean particle diameter slightly larger than 3 μm . By increasing the speed of agitation during the stabilization process and the length of stabilization in Method F, it was possible to produce microspheres with a mean diameter of 2.23 μm with a more homogeneous microsphere population. In method G, the protein concentration was decreased, and for the same reason as for thermal-denatured microsphere preparation, smaller microspheres were produced with a mean diameter of 2.02 μm .

TABLE 2.7 *Mean Particle Size Obtained With Some Variations Of The Methods Cited In The Literature Used To Prepare Glutaraldehyde Cross-Linked Albumin Microspheres*

PREPARATION METHOD	MEAN SIZE (μm)
Yapel, (1985)	10 - 80*
Ratcliffe <i>et al.</i> , (1984)	3.30 \pm 1.70†
E	3.12 \pm 2.45 ⁺
F	2.23 \pm 0.90 ⁺
G	2.02 \pm 0.62 ⁺

* No other information available

† Geometric mean particle size diameter and standard deviation, measured by Coulter Counter

⁺ Geometric mean particle size diameter and standard deviation, manually measured from SEM photomicrographs (n \geq 100)

Radiolabelled microspheres were prepared using method G, with the added incorporation of [¹²⁵I]-BSA during preparation. This had no effect on the subsequent size distributions and mean diameters of radiolabelled microspheres prepared under the same experimental conditions, and the method was reproducible (n \geq 3). Examination of radiolabelled microspheres by SEM revealed smooth, spherical particles. Assessment of free [¹²⁵I]-BSA remaining in the microsphere preparations after 4 and 24 hr incubation in PBS showed, in both cases, that less than 1.5% of the total radioactivity of each microsphere sample was unassociated with the microspheres. This indicates that \geq 98% of the radioactivity was incorporated within the matrix of the microspheres.

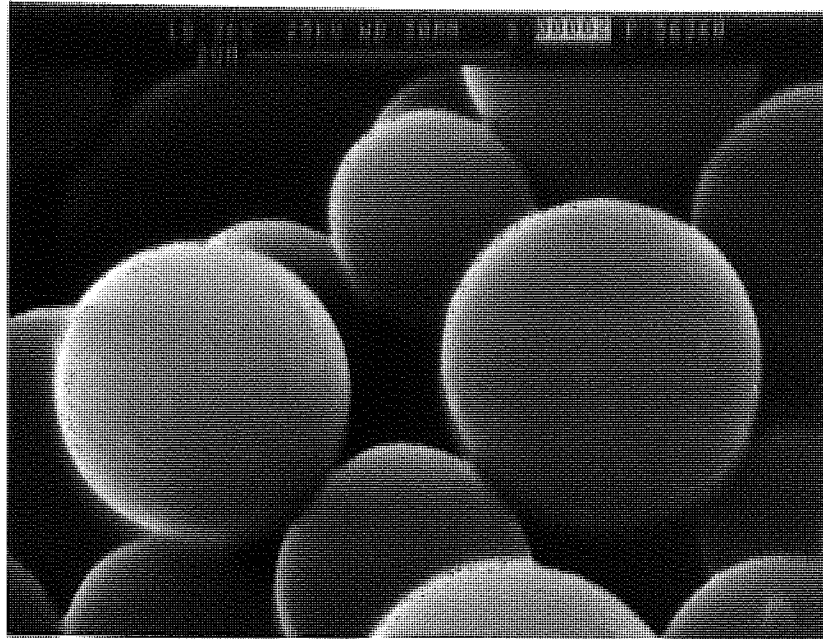


PLATE 2.2 *Scanning Electron Micrograph Of Chemically Cross-Linked Albumin Microspheres*

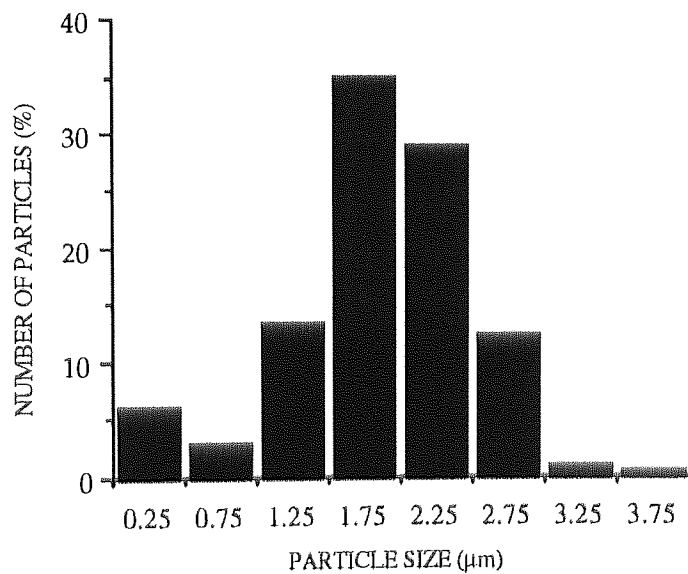


FIGURE 2.2 *Particle Size Distribution Of Chemically Cross-Linked Albumin Microspheres Prepared By Method G. Geometric Mean Diameter = 2.02 μm; $\sigma_g = 0.62 \mu\text{m}$*

2.5.2 PLA MICROSPHERE PREPARATION

Method I was the preparation conditions chosen for the production of PLA microspheres. SEM examination of prepared microsphere batches showed smooth, spherical particles (Plate 2.3). Particle diameters were measured manually, with ≥ 100 microspheres being measured for each batch. Each batch data was represented in the form of a frequency size distribution curve, which repeatedly gave rise to slightly skewed size distribution curves, an example of which is shown in Figure 2.3, along with the calculated geometric mean and σ_g . The results were reproducible for batches of microspheres prepared under the same experimental conditions ($n \geq 3$).

Table 2.8 shows some of the mean particle sizes, measured by SEM, achieved for PLA microspheres using methods based on the original method of Beck *et al.*, (1979). An increase in the rate of stirring during microsphere preparation was found to reduce mean particle diameter for both PLA polymers employed. Again, the faster the stirring rate, the finer the droplets that are formed in the emulsion, and consequently the smaller the mean particle diameter.

With respect to the molecular weights of PLA used, the results are similar to those of Wichert & Rohdewald (1990), in that an increase in PLA molecular weight leads to an increase in mean particle size. The viscosity of PLA (2,000 Da Mwt) in dichloromethane is lower than that of PLA (57,000 Da Mwt). This increase in viscosity of the internal phase with the higher Mwt PLA, results in the formation of larger droplet sizes during homogenization, and subsequently to larger particle diameters. A similar effect is observed when the polymer concentration in the organic phase is increased (Watts *et al.*, 1990). The increased velocity of the effect on viscosity is boosted by the lowering of the temperature of both phases ($\leq 4^\circ\text{C}$) prior to microsphere formation.

TABLE 2.8 Mean Particle Size Obtained With Some Variations Of The Methods Cited In The Literature Used To Prepare PLA Microspheres

PREPARATION METHOD	MEAN SIZE (μm)
Benita <i>et al.</i> , (1984a)	$58.2 \pm 15.6^*$
Wichert & Rohdewald, (1990)	$\leq 10^\dagger$
H	$4.03 \pm 2.25^+$
I	$2.53 \pm 1.59^+$
J	$4.36 \pm 3.22^+$
K	$3.45 \pm 2.01^+$

*Measured manually by SEM photomicrographs

†Measured manually by SEM photomicrographs, particle mean diameter expressed by volume diameter

+Geometric mean particle size diameter and standard deviation, manually measured from SEM photomicrographs ($n \geq 100$)

2.5.3 ADSORPTION OF POLOXAMINE 908 ONTO CHEMICALLY CROSS-LINKED ALBUMIN MICROSPHERES

In order to determine whether a coating of Poloxamine 908 was adsorbed onto the surface of chemically cross-linked albumin microspheres, the surface charge of glutaraldehyde cross-linked albumin microspheres soaked in Poloxamine 908 solutions were measured in terms of their zeta potential values, the results of which are shown in Figure 2.4. All albumin microsphere formulations were less negatively charged than the Polyscience latex microspheres. Coating of albumin microspheres in 2.0% and 0.2% Poloxamine 908 solutions for 30 min at room temperature showed a decrease in surface charge from -19.4 mV to -1.24 mV and -3.4 mV respectively. This suggests the adsorption of the block co-polymer onto the surface of the albumin microspheres. This would indicate the albumin microspheres have a hydrophobic surface, onto which the hydrophobic blocks of the copolymer are physically adsorbed, whilst the hydrophilic parts are protruding into the surrounding media, creating a sterically stabilized hydrophilic surface (Figure 2.5).

It has long been known that the surface charge on particulates has an effect on the biodistribution and clearance rate of intravenously administered microspheres (Wilkins & Myers, 1966). Tabata & Ikada (1990), reported that macrophage phagocytosis of

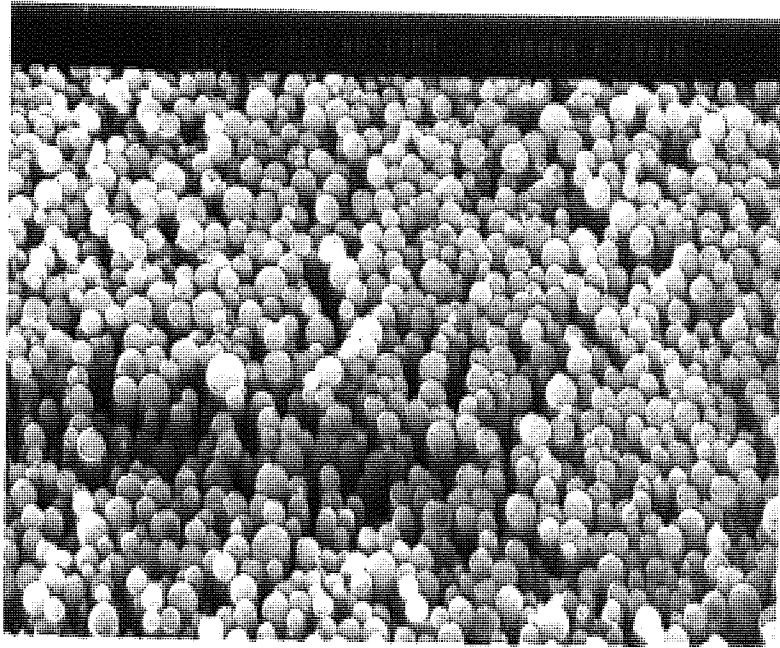


PLATE 2.3 *Scanning Electron Micrograph Of PLA Microspheres*

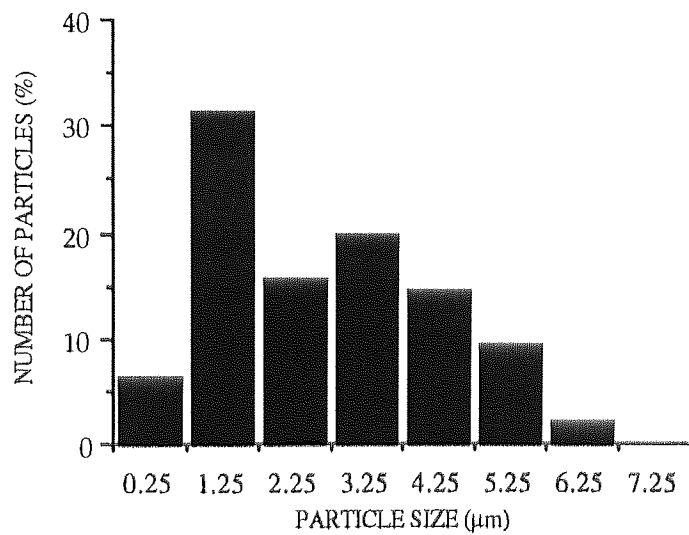


FIGURE 2.3 *Particle Size Distribution Of PLA Microspheres Prepared By Method I. Geometric Mean Diameter = 2.53 μm; $\sigma_g = 0.159 \mu\text{m}$*

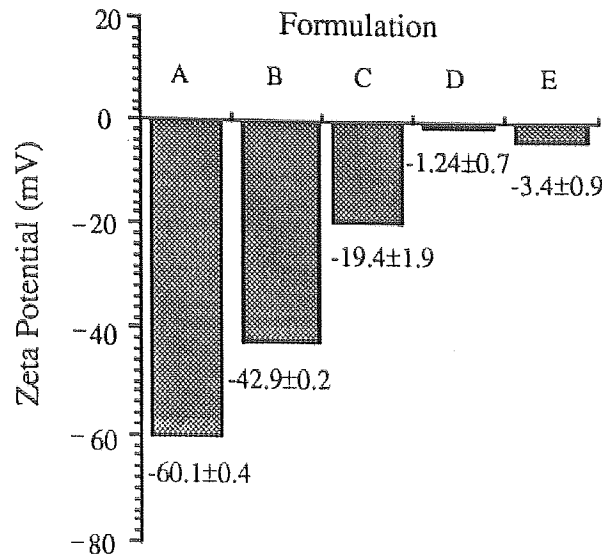


FIGURE 2.4 *Surface Charge Of Various Formulations Of Chemically Cross-Linked Albumin Microspheres Coated With Poloxamine 908 Measured As Zeta Potential (mean \pm sd; n = 3)*
 A) AZ55 Standard Latex Particles In AZ55 Buffer, B) Polyscience Latex Microspheres, C) Glutaraldehyde Cross-Linked Albumin Microspheres, D) Glutaraldehyde Cross-Linked Albumin Microspheres Coated With Poloxamine 908 (2.0% w/v), E) Glutaraldehyde Cross-Linked Albumin Microspheres Coated With Poloxamine 908 (0.2% w/v). B-D 37 mM Phosphate Buffered Saline, pH 7.2

cellulose particles is enhanced as the zeta potential increases for both negatively charged and positively charged surfaces, the lowest phagocytosis being realized for the surface with a zeta potential of zero. The same group also found that microspheres with hydrophobic surfaces were more readily phagocytosed than those with hydrophilic surfaces (Tabata & Ikada, 1988). Thus, it would appear that adsorption of Poloxamer 908 on the surface of albumin microspheres not only decreases the negative surface charge of the microspheres but will also reduce their hydrophobicity, and therefore, the coated albumin microspheres may be less readily phagocytosed by cells of the MPS *in vivo*. This has been shown for Poloxamine 908-coated polystyrene microspheres (60 nm mean diameter) by Illum *et al.*, (1987), who reported 60% of the initial Poloxamer 908-coated microspheres dosed remained in the blood 1 hour post intravenous administration, compared to only 3% of non-coated microspheres.

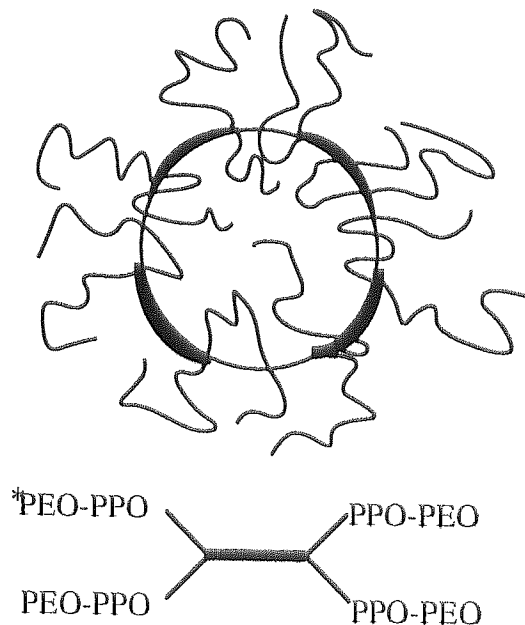


FIGURE 2.5 *The Structure Of Poloxamine 908 And A Model For Their Adsorption To Particles (adapted from Wright & Illum, 1992)*
PEO - Polyoxyethylene, PPO - Polyoxypropylene
**Molecular Blocks (Average value in moles) PEO-PPO 122-22*

2.6 SUMMARY

Albumin microspheres were prepared by both thermal-denaturation and chemical cross-linking methods of microsphere stabilization. PLA microspheres were prepared by the solvent evaporation/emulsification method. Microspheres were produced in free-flowing powders without particle aggregation and coalescence. Measurement of particle size from SEM photomicrographs gave rise to slightly skewed frequency size distribution plots. Conversion to log-normal frequency size distributions enabled particle size to be measured in terms of geometric mean and standard deviation for each microsphere batch. Microspheres were routinely produced with mean particle diameters around 2.5 μm . [^{125}I]-labeled albumin microspheres were produced for use in *in vivo* tissue distribution studies. Chemically cross-linked albumin microspheres were coated with Poloxamer 908, giving rise to a reduction in the negative surface charge on the microspheres from -19.4 mV to -1.24 mV as measured by zeta potential analysis.

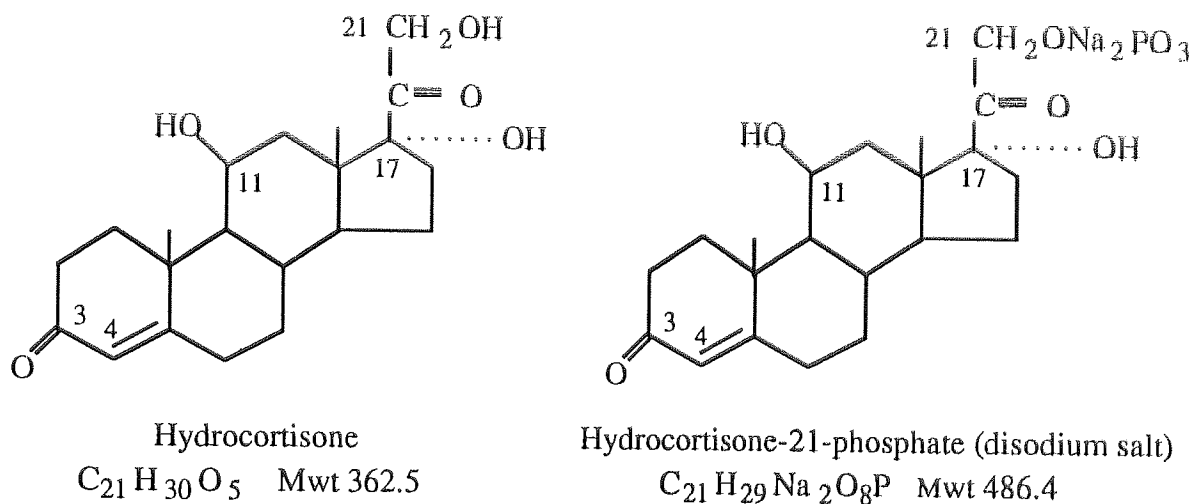
CHAPTER 3

DEVELOPMENT OF A HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE SIMULTANEOUS, QUANTITATIVE ANALYSIS OF HYDROCORTISONE AND HYDROCORTISONE-21-PHOSPHATE

3. DEVELOPMENT OF A HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE SIMULTANEOUS, QUANTITATIVE ANALYSIS OF HYDROCORTISONE AND HYDROCORTISONE-21-PHOSPHATE

An accurate method to simultaneously quantify hydrocortisone (HC) and hydrocortisone-21-phosphate disodium salt (H-21-P), the structures of which are shown in Figure 3.1, was required for the determination of drug-loading levels and drug-release profiles of steroid-incorporated albumin and PLA microspheres. High-performance liquid chromatography (HPLC) was the assay procedure chosen, based on its ability to separate and accurately quantify molecules of similar structures and properties. A method was developed based on a reversed-phase (RP), ion-pairing approach, and this is outlined in Section 3.4 after the principles of HPLC have been briefly discussed.

FIGURE 3.1 The Chemical Structures Of Hydrocortisone And Hydrocortisone-21-Phosphate Disodium Salt (adapted from Florey, 1983)

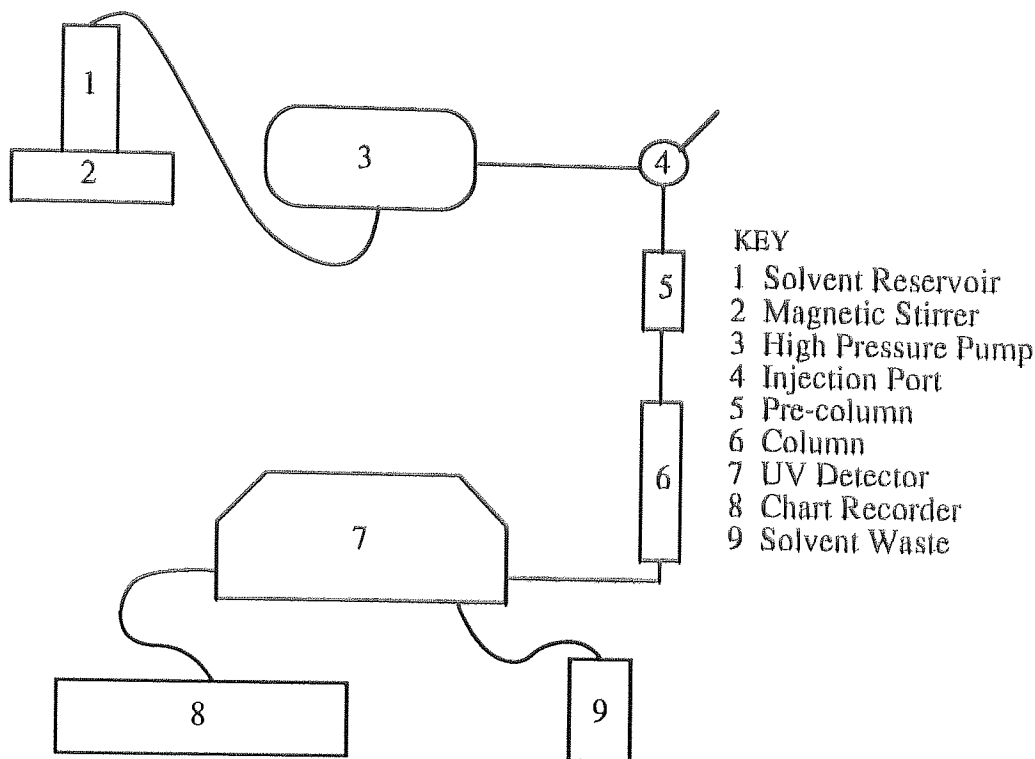


3.1 AN INTRODUCTION TO HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

HPLC was developed in the late 1960s from a knowledge of the theoretical principles which had already been established for column chromatography. Comprehensive accounts of the theoretical principles and practical aspects of HPLC may be found in several texts and reviews (Braithwaite & Smith, 1985; Kirschbaum, 1989; Munson, 1984). Very simply, the system involves the delivery of a mobile phase (MP) through an injection valve onto a solid phase chromatographic column, thereby carrying the sample through onto the column. A combination of partitioning and adsorption

processes of analytes between the solid stationary phase and liquid MP, by nature of their physicochemical characteristics, results in separation and elution of analytes onto a detection device where they may be quantified. A basic HPLC system is illustrated diagrammatically in Figure 3.2.

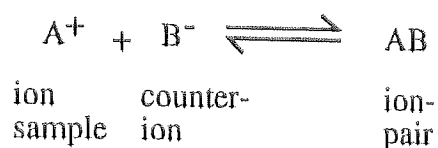
FIGURE 3.2 *The Basic Components Of A High-Performance Liquid Chromatography System (adapted from Li Wan Po & Irwin, 1980)*



Since an HPLC assay system was required to simultaneously quantify HC, a relatively non-polar compound, and H-21-P an ionic compound, a RP stationary phase was chosen. RP systems allow polar, non-polar, ionic and ionizable compounds to be effectively chromatographed simultaneously, using a single column and MP. In RP partition chromatography, the stationary phase is less polar than the MP, and consequently the solutes are eluted in order of their decreasing polarity. The stationary phase is usually silica, chemically bonded through a siloxane (Si-O-Si) linkage to a low polar functional group. These phases are prepared by treating the surface silanol groups with an organosilane reagent; for octadecylsilane (ODS, $C_{18}H_{37}Si-$) bonded phases, the reagent used is octadecyltrichlorosilane. The reaction is followed by the hydrolysis of the residual chlorines and the resulting material is a silica base with a coating of ODS groups on the surface. Untreated silanol groups may be 'capped' by treatment with trimethylchlorosilane to eliminate adsorption effects.

The MP in RP systems generally comprises water and a less polar organic solvent modifier, *eg.* methanol, acetonitrile. Separations in these systems are considered to be due to different degrees of hydrophobicity of the solutes; the less polar solutes partitioning to a greater extent into the non-polar stationary phase, and consequently being retained on the column for longer than the more polar solutes. The rate of elution is increased by reducing the polarity of the MP. This simple alteration allows the rate of elution of the solutes to be adjusted to an optimum which may be expressed in terms of various mathematical parameters.

As mentioned previously, RP systems may be used to chromatographically separate non-polar and ionic compounds. This may be achieved by using ion-pair partition chromatography. This form of chromatography involves the use of a MP system which contains a counter-ion with the opposite charge to that of the ion(s) to be analysed, and which will form an ion-pair with the ionic sample components:



Counter-ions usually contain bulky organic substitutes so that the ion-pair subsequently formed will be hydrophobic in character. This enables conventional RP packings and methanol-water based MP systems to be used for ionic samples. The great utility of this technique is in its efficiency in the analysis of mixtures containing ionic and non-ionic analytes.

The principal advantages of HPLC are its ability to efficiently separate and detect a wide range of molecules with improved resolution, faster separation times and with increased accuracy, precision and sensitivity. The wide applicability of HPLC has resulted in it becoming one of the most popular techniques being used in clinical and pharmaceutical analytical work, being especially useful in that biological fluids and serum may be applied to the column, after passage through a pre-column, for the separation and measurement of drugs and their metabolites. Reviews on the applications of HPLC (Irwin & Scott, 1982; Li Wan Po & Irwin, 1980), as well as texts devoted to the analysis of steroids by HPLC (Görög, 1983), indicate the use of this technique for steroid quantitation both in pharmaceutical formulations and pharmacokinetic studies.

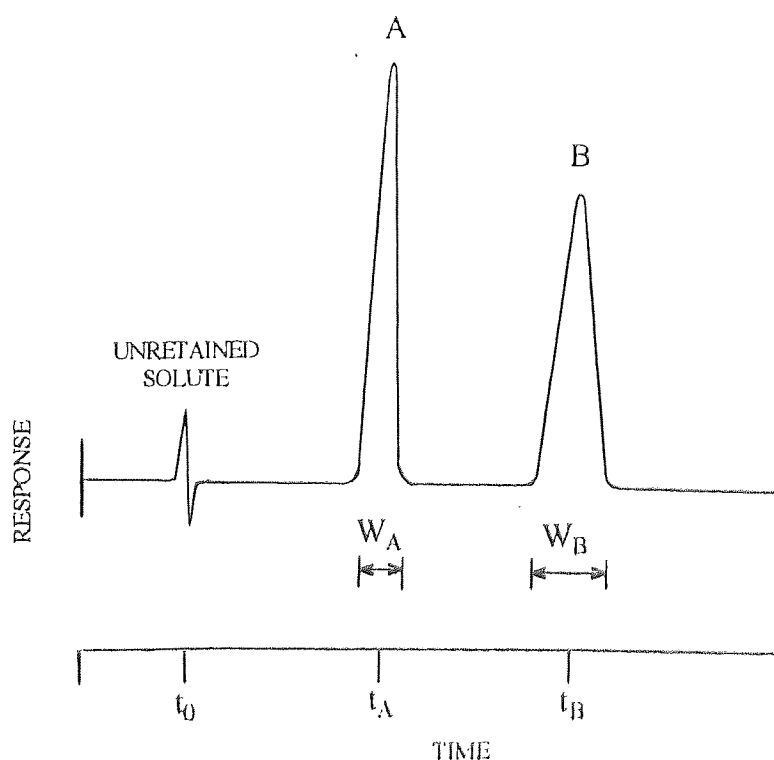
The development of an HPLC system may be achieved by the adjustment of many variables including the choice of stationary phase, MP composition and UV wavelength

for optimum analyte detection. Various mathematical parameters may be used to judge the system's performance with respect to these variables and this is discussed below. The inclusion of an internal standard in the sample aids standardization by minimizing variance caused by injection volume. Analyte concentrations are determined by interpolation from calibration plots which are validated statistically in terms of their linearity.

3.1.1 MATHEMATICAL PARAMETERS

A number of mathematical parameters may be defined in order to quantify and improve the chromatographical performance of an assay system in terms of retention and resolution of analytes. Figure 3.3 illustrates a typical HPLC chromatogram for a two component mixture where t_A and t_B are the retention times of components A and B respectively, and t_0 is the retention time of any unretained solute.

FIGURE 3.3 *A Typical Chromatogram For A Two Component Separation Where t_A And t_B Are The Retention Times Of Components A And B Respectively, And t_0 Is The Retention Time Of Any Unretained Solute (adapted from Li Wan Po & Irwin, 1980)*



The column capacity factor, k' , is a measure of the sample retention and is probably the most useful separation parameter. It may be calculated using the formula;-

$$k_A' = (t_A - t_0) / t_0$$

For good, isocratic separation, k' should be in the range 1 to 10. Small capacity factors of less than one indicate inadequate separation from the solvent, whilst those of greater than 10 are associated with long retention times and broad peaks.

The number of theoretical plates, N , gives a measure of column efficiency which is dependent on the degree of band broadening relative to the time taken to elute the analyte. This may be defined by the equation;-

$$N = 16 (t_A / W_A)^2 \quad \text{where } W_A = \text{base width of peak A in mm}$$

N is usually expressed per metre of column length (derived from dividing the above formula by the column length in metres) and for typical systems should be in the range of 2500-10000. Columns with large N values will produce narrow peaks and better resolutions than those with lower N values.

The column efficiency may also be expressed as the height equivalent of a theoretical plate, H , using the following formula;-

$$H = L / N \quad \text{where } L = \text{column length in mm}$$

Values of H should lie between 25-100 μm .

The resolution, R_S , is a measure of the efficiency of separation of different components and may be represented by the equation;-

$$R_S = 2 (t_B - t_A) / (W_A + W_B)$$

An R_S of 1.0 indicates a satisfactory separation with about 2% overlap whilst a value of 1.5 indicates almost total separation.

3.2 MATERIALS

All chemicals and solvents were purchased from appropriate chemical suppliers as listed in Appendix I. HPLC grade solvents and water were used for MP preparations. All other chemicals were either of analar or reagent grade. Distilled water was used throughout for the preparation of analyte solutions.

3.3 INSTRUMENTATION

3.3.1 ANALYTICAL

Ultra-violet (UV) absorption readings and spectrum profiles were measured using a Cecil C292 Digital UV Spectrophotometer and a Cecil 594 Double Beam Spectrophotometer (Cecil Instruments, Cambridge, England) respectively. In both cases, 10 mm quartz cells (Merck, Darmstadt, Germany) were employed.

HPLC analyses were performed using a system constructed from a number of basic components. A Gilson (Villiers-le-Bel, France) dual reciprocating pump delivered mobile phases typically at a flow rate of 0.65 ml min^{-1} with a Gilson Manometric Module 802 (Villiers-le-Bel, France) monitoring pressure with typical values between 500-1000 psi. A LiChrospher® 100 (Merck, Darmstadt, Germany) RP-18 (particle size $5 \mu\text{m}$) pre-column (4 mm x 4 mm; internal diameter x column length [ID x CL]) was placed before a LiChrospher® 100 RP-18 (particle size $5 \mu\text{m}$) endcapped stainless steel column (125 mm CL x 4 mm ID). Both columns were held in LichroCART® 4 mm x 4 mm and 125 mm x 4 mm ID x CL HPLC cartridges respectively. Samples were introduced through a Rheodyne 7120 injection valve fitted with a $20 \mu\text{l}$ loop, and UV detection at 242 nm was achieved with a Cecil CE212 Variable Wavelength Monitor (Cecil Instruments, Cambridge, England). Chromatograms were recorded using a Gallenkamp Chart Recorder (Houston Instruments, Belgium) operated at a speed of 0.83 mm min^{-1} .

3.3.2 SUBSIDIARY

All pH measurements were undertaken using a WPA CD 660 Digital pH meter (WPA Linton, Cambridge, England) appropriately calibrated with ColourKey Buffer Solutions (BDH Ltd). The following Sartorius electronic balances (Sartorius GMBH, Göttingen, Germany) were employed for accurate weighing purposes; an analytical A200S (4 decimal places) and a research R200D (5 decimal places). A Kerry

Laboratory Sonicator was employed to degas MP preparations and as an aid to dissolution.

3.4 EXPERIMENTAL

3.4.1 DETERMINATION OF OPTIMUM UV WAVELENGTH FOR ANALYTE DETECTION

The optimum UV wavelength setting for detection of HC and H-21-P elution from the column was determined by carrying out UV absorbance spectrum analyses. UV absorbance spectra were also measured for candidate internal standard compounds, namely cortisone, prednisolone, methyl-paraben and propyl-paraben. Solutions of HC, H-21-P, and the four internal standard candidates were each made in methanol at a concentration of $1 \mu\text{g ml}^{-1}$, diluted appropriately, and their UV spectrum from 190-350 nm measured. The UV absorbance profiles of HC, H-21-P, and internal standard candidates are shown in Figure 3.4. The maximum UV absorbance wavelengths for these, and each internal standard candidate, together with their calculated extinction coefficients and molar extinction coefficients at 242 nm in methanol, are shown in Table 3.1.

FIGURE 3.4 *UV Absorbance Profiles Of Hydrocortisone, Hydrocortisone-21-Phosphate And Internal Standard Candidates In Methanol Between 190-350 nm*

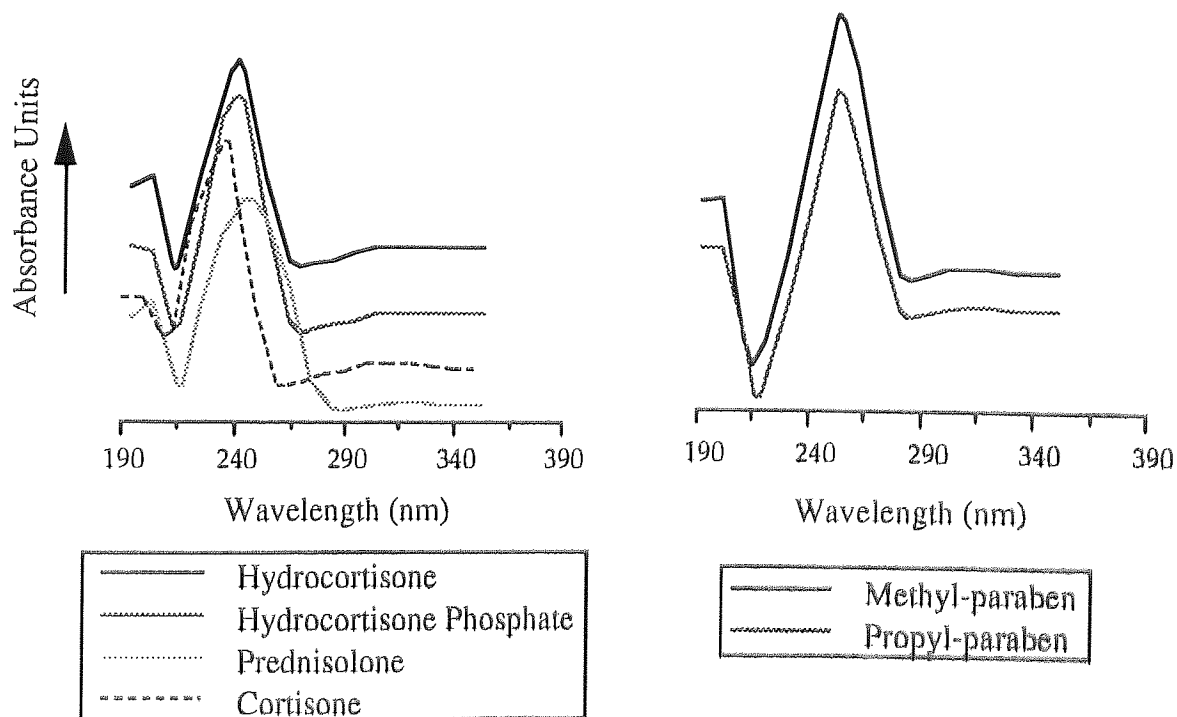


TABLE 3.1 *Maximum UV Absorbance Wavelengths And Extinction Coefficients At 242 nm For Hydrocortisone, Hydrocortisone-21-Phosphate And Candidate Internal Standard Compounds In Methanol*

COMPOUND	MAXIMUM ABSORBANCE WAVELENGTH (nm)	EXTINCTION COEFFICIENT $E_{1cm}^{1\%}$	MOLAR EXTINCTION COEFFICIENT ϵ
HYDROCORTISONE	242	442.0	16,000
HYDROCORTISONE -21-PHOSPHATE	242	294.2	14,300
CORTISONE	238	727.0	26,200
PREDNISOLONE	244	616.0	22,200
METHYL-PARABEN	258	1065.8	16,200
PROPYL-PARABEN	256	638.9	11,500

The optimum UV wavelength setting for HPLC analysis of HC and H-21-P in methanol was at 242 nm. All four internal standard candidate compounds absorbed UV light at this wavelength and were hence suitable as internal standard candidates under HPLC running conditions using a wavelength of analyte detection set at 242 nm.

3.4.2 DETERMINATION OF MOBILE PHASE COMPOSITION FOR SEPARATION OF HYDROCORTISONE AND HYDROCORTISONE-21-PHOSPHATE

HC and H-21-P were simultaneously analysed by HPLC using MPs composed of various concentrations of methanol (45-65% v/v) in water. In all cases, H-21-P was unretained on the column, having a retention time of t_0 . Since H-21-P, an ionic molecule, was not retained on the column using a MP composed solely of methanol and water, diethylamine was added to act in a similar manner to an ion-pairing agent. Usually, ion-pairing agents are ionic compounds containing a counter-ion with the opposite charge to that of the analyte ion. These interact and form a more hydrophobic ion-pair molecule. This has the result of retaining the analyte on the column for a

longer period of time. The negatively charged phosphate ion of the H-21-P molecule interacts with the positively charged nitrogen ion of diethylamine molecule forming an ion-pair. Thus, the H-21-P is retained for a longer period on the column. Thus, diethylamine (final concentration 5 mM) was added to each MP composition, the addition of which resulted in an increase in pH of each MP to >pH 10. Due to instability of the bonded phase at such high pH values, the pH of each mobile phase was adjusted to pH 3.5 with orthophosphoric acid. A flow rate of 0.65 ml min⁻¹ was employed and the column elutants recorded spectrophotometrically at 242 nm with a sensitivity of 0.1 AUFS. Sample injection loop volume was 20 µl. From the resulting chromatograms it could be seen that the addition of diethylamine to the MP acted to retain the H-21-P on the column, increasing its retention time and giving capacity factors greater than one.

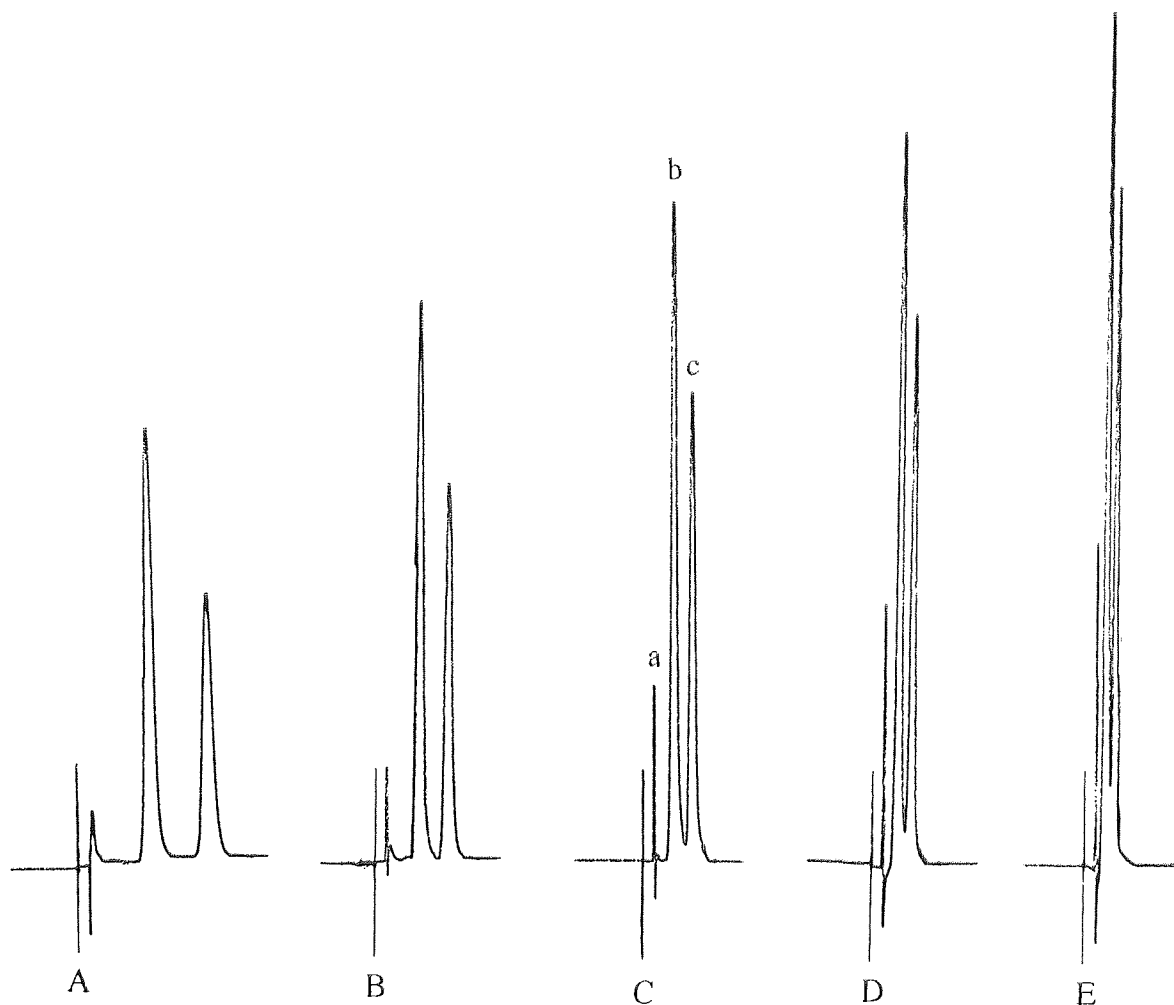
The effect of varying methanol content on the retention and resolution of HC and H-21-P was investigated, keeping the pH and concentration of diethylamine in each MP constant. The resulting chromatograms for each solvent system along with the calculated values of t_r , k' , N , H and R_s are shown in Figure 3.5 and Table 3.2 respectively.

From the chromatograms and the mathematical parameters it may be seen that the optimum methanol concentration in the mobile phase for the separation of HC and H-21-P is 45% v/v. However, in subsequent analyses of these two compounds, 55% v/v methanol was used, since, with this solvent composition, there was still adequate compound resolution but the lower retention times allowed the total run time for each sample to be reduced. Since the addition of diethylamine retained the H-21-P on the column, giving rise to a retention time of 4.8 min with a methanol composition of 55% v/v in water, no further attempts were made to investigate other diethylamine concentrations or any other ion-pairing agents. Similarly, with an adequate separation of HC and H-21-P using a methanol composition of 55% v/v and 5mM diethylamine adjusted to pH 3.5, no further attempts were made to investigate further the effect of pH on HC and H-21-P separation.

3.4.3 STUDY OF INTERNAL STANDARD CANDIDATES

Once an adequate assay system for the simultaneous separation of HC and H-21-P was achieved, the internal standard candidates were analysed with HC and H-21-P simultaneously, to determine which would be resolved under these conditions.

FIGURE 3.5 *The Effect On Hydrocortisone And Hydrocortisone-21-Phosphate Chromatographic Separation By Increasing The Percentage Of Methanol In The Mobile Phase From 45-65% v/v In Water*



PEAK IDENTITY:

(a) solvent front; (b) hydrocortisone-21-phosphate; (c) hydrocortisone

MOBILE PHASE COMPOSITION:

(A) MeOH 45%; (B) MeOH 50%; (C) MeOH 55%; (D) MeOH 60% (E) MeOH 65%
All compositions contained 5mM diethylamine and were adjusted to pH 3.5 with orthophosphoric acid. All MP systems were degassed by sonication prior to use.

HPLC CONDITIONS:

Column LiChrospher® 100 (5 µm pore diameter) 125 mm x 4 mm CL x ID
Flow rate 0.65 ml min⁻¹; Loop size 20 µl; Chart speed 0.83 mm min⁻¹;
Wavelength 242 nm; Sensitivity 0.1 AUFS

TABLE 3.2 *The Effect Of Increasing The Percentage Of Methanol In The Mobile Phase† On The Simultaneous Analysis Of Hydrocortisone And Hydrocortisone-21-Phosphate By HPLC* In Terms Of Retention Time, Chromatographic Efficiency And Compound Resolution*

PARAMETER	% METHANOL IN MOBILE PHASE	HYDROCORTISONE-21-PHOSPHATE	HYDROCORTISONE
RETENTION TIME t_r (min)	45	12.0	20.4
	50	7.2	12.0
	55	4.8	7.8
	60	4.2	6.0
	65	3.0	3.6
CAPACITY RATIO k'	45	4.0	7.5
	50	2.0	4.0
	55	1.7	3.3
	60	1.3	2.3
	65	0.7	1.0
NUMBER OF THEORETICAL PLATES N (m^{-1})	45	1672	4833
	50	1070	2973
	55	1070	2826
	60	1457	1672
	65	1672	1070
HEIGHT EQUIVALENT TO A THEORETICAL PLATE H (μm)	45	75	26
	50	117	42
	55	117	44
	60	86	75
	65	75	117
RESOLUTION R_s	45	1.77	
	50	1.28	
	55	1.00	
	60	0.80	
	65	0.60	

†MOBILE PHASE COMPOSITION:

All compositions contained 5mM diethylamine and were adjusted to pH 3.5 with orthophosphoric acid. All MP systems were degased by sonication prior to use.

*HPLC CONDITIONS:

Column LiChrospher® 100 (5 μm pore diameter) 125 mm x 4 mm CL x ID

Flow rate 0.65 ml min^{-1} ; Loop size 20 μl ; Chart speed 0.83 mm min^{-1} ;

Wavelength 242 nm; Sensitivity 0.1 AUFS

Solutions of cortisone, prednisolone, methyl paraben and propyl paraben in methanol were each injected onto the column using the chosen MP and HPLC system conditions for HC and H-21-P separation outlined in Section 3.4.2. The retention time for each compound is shown in Table 3.3.

All candidates exhibited narrow chromatographic peaks suitable for peak height measurements. However, only propyl paraben exhibited a suitable retention time, that of 12 minutes, which would be eluted after H-21-P ($t_r = 4.8$ min) and HC ($t_r = 7.8$ min) separation under the HPLC conditions stated. Hence propyl paraben was used as the internal standard for statistically validating all quantitative measurements.

TABLE 3.3 *Retention Times Of Internal Standard Candidate Compounds Analysed Simultaneously With Hydrocortisone And Hydrocortisone-21-Phosphate By HPLC*†*

CANDIDATE INTERNAL STANDARD COMPOUND	RETENTION TIME ON COLUMN (min)
CORTISONE	6.6
PREDNISOLONE	8.4
METHYL PARABEN	4.2
PROPYL PARABEN	12.0

* HPLC CONDITIONS:

Column LiChrospher® 100 (5 µm Pore Diameter) 125 mm X 4 mm CL X ID; Flow Rate 0.65 ml min⁻¹; Loop Size 20 µl; Chart Speed 0.83 mm min⁻¹; Wavelength 242 nm; Sensitivity 0.1 AUFS

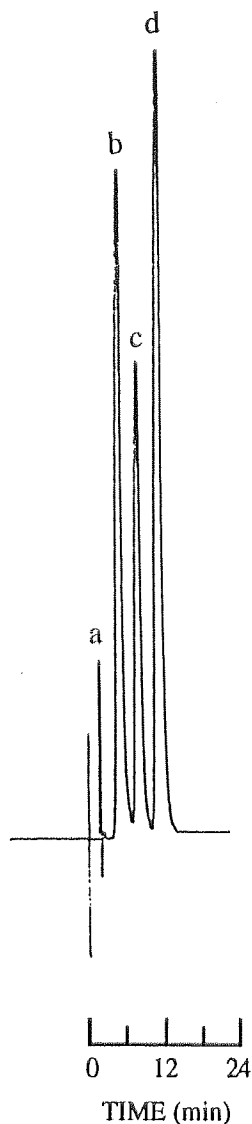
† MOBILE PHASE COMPOSTITION:

55% v/v Methanol In Water, 5 mM Diethylamine, Adjusted To pH 3.5 With Orthophosphoric Acid, Degased By Sonication Prior To Use.

3.4.4 QUANTITATIVE ANALYSIS OF HYDROCORTISONE AND HYDROCORTISONE-21-PHOSPHATE

Quantitation of sample drug concentrations were to be based on peak height ratios using a constant concentration of internal standard. A series of calibration solutions were made in methanol using HC and H-21-P concentrations between 10-80 µg ml⁻¹ with propyl paraben as internal standard at a concentration of 40 µg ml⁻¹.

FIGURE 3.6 *A Typical Chromatogram For The Separation Of Hydrocortisone And Hydrocortisone-21-Phosphate With Propyl Paraben As An Internal Standard*



PEAK IDENTITY:

(a) unretained solute; (b) hydrocortisone-21-phosphate; (c) hydrocortisone;
(d) propyl paraben

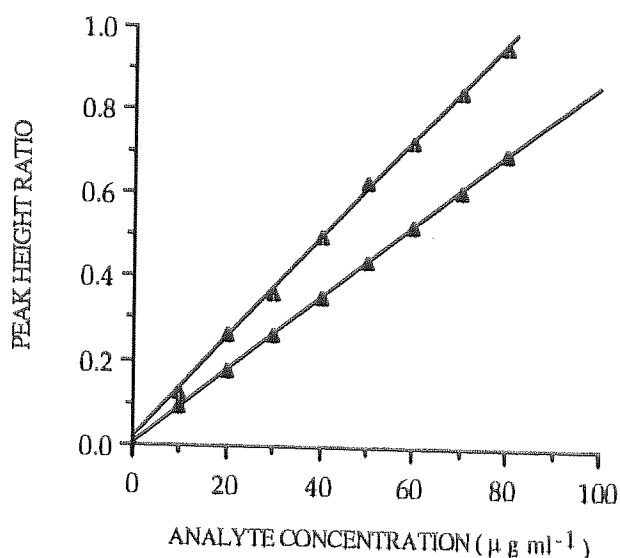
MOBILE PHASE COMPOSITION:

55% v/v methanol in water, 5 mM diethylamine, adjusted to pH 3.5 with orthophosphoric acid, degassed by sonication prior to use

HPLC CONDITIONS:

Column LiChrospher® 100 (5 µm pore diameter) 125 mm x 4 mm CL x ID
Flow rate 0.65 ml min⁻¹; Loop size 20 µl; Chart speed 0.83 mm min⁻¹; Wavelength 242 nm; Sensitivity 0.1 AUFS; Internal standard propyl paraben (40 µg ml⁻¹)

FIGURE 3.7 *A Typical Calibration Plot For Hydrocortisone (\blacktriangle) And Hydrocortisone-21-Phosphate (\triangle) Quantitative Analysis By HPLC*† Using Propyl Paraben As An Internal Standard*



*HPLC CONDITIONS:

Column LiChrospher® 100 (5 µm pore diameter) 125 mm x 4 mm CL x ID
 Flow rate 0.65 ml min⁻¹; Loop size 20 µl; Chart speed 0.83 mm min⁻¹; Wavelength 242 nm; Sensitivity 0.1 AUFS; Internal standard propyl paraben (40 µg ml⁻¹)

†MOBILE PHASE COMPOSITION:

55% v/v methanol in water, 5 mM diethylamine, adjusted to pH 3.5 with orthophosphoric acid, degassed by sonication prior to use

TABLE 3.4 *Corresponding Calibration Statistics For Figure 3.7*

ANALYTE	SLOPE	INTERCEPT	CORRELATION COEFFICIENT
HYDROCORTISONE	8.732×10^{-3}	2.429×10^{-3}	1.000
HYDROCORTISONE-21-PHOSPHATE	1.205×10^{-2}	1.211×10^{-2}	0.999

A typical chromatogram of a calibration standard is shown in Figure 3.6. By measuring the peak heights of a series of known drug concentrations and dividing each by the height of its corresponding internal standard peak height, the peak height ratios were calculated. These ratios were plotted against their corresponding drug concentrations and the linearity of the plots examined statistically to assess the validity of measuring unknown drug concentrations by the same method. Typical calibration plots of peak height ratios against corresponding drug concentrations for both HC and H-21-P are shown in Figure 3.7. The corresponding calibration statistics for the plots are shown in Table 3.4. Since both drug calibration series show a high degree of statistical linearity it may be taken that unknown concentrations of these drugs in sample preparations analysed under identical HPLC conditions may be calculated.

3.5 SUMMARY

A precise method to simultaneously and quantitatively analyse samples containing both HC, H-21-P and an internal standard, propyl-paraben was developed using a reversed-phase HPLC assay system. The method developed used the following MP composition; 55% v/v methanol in water with the addition of 5mM diethylamine, and was adjusted to pH 3.5 with orthophosphoric acid. The following HPLC conditions were used, namely a LiChrospher® 100 (5 µm Pore Diameter) 125 mm x 4 mm CL x ID column, with MP being delivered at a flow rate of 0.65 ml min⁻¹. The sample injector loop size was 20 µl, with eluted analytes being detected by UV spectrophotometry at 242 nm.

The method developed, as a technique for quantitative analysis of the two steroids, was validated in terms of linearity of calibration plots with respect to analyte concentration. The linear regression correlation coefficients of these plots were determined to be ≥ 0.999 , thus validating the assay procedure for each of the steroid compounds. The assay procedure was thus employed to precisely determine drug-loading levels and drug-release profiles of steroid-incorporated albumin and PLA microspheres.

CHAPTER 4

STEROID-INCORPORATED ALBUMIN AND POLYLACTIC ACID
MICROSPHERES

4. STEROID-INCORPORATED ALBUMIN AND POLYLACTIC ACID MICROSPHERES

4.1 INTRODUCTION

For a drug delivery system to be effective in alleviating a disease it is essential that the minimum effective concentration of medication is attained at the target site (Friend & Pangburn, 1987). In the case of particulate carriers this depends on their drug content (Davis, 1986). An increase in drug entrapment in microspheres would reduce the amount of carrier required to deliver a specified dose of the drug, and hence improve its target tissue uptake at the capillary bed (Oppenheim *et al.*, 1984). Therefore, it is imperative to design microspheres with high levels of drug entrapment.

Various steroids have been incorporated into albumin microspheres. Lee *et al.*, (1981), investigated the potential of progesterone-loaded albumin microspheres as an injectable, contraceptive device. Glutaraldehyde cross-linked albumin microspheres (mean diameter 100-200 μm) with a progesterone loading of 5-30% w/w were prepared. *In vivo* experiments showed the sustained release of progesterone over a period of 20 days after intramuscular injection in rabbits. Burgess *et al.*, (1987), investigated the potential use of prednisolone-loaded albumin microspheres (20% w/w prednisolone, mean diameter 23 μm) as an intra-articularly administered, sustained release system, in an attempt to retain prednisolone within the synovial joint. *In vivo* studies in New Zealand white rabbits indicated drug-incorporated microsphere preparations had sustained release characteristics, when compared to control microcrystalline suspensions of the drug (Burgess & Davis, 1988). Saunders *et al.*, (1991), also reported the incorporation of anti-inflammatory drugs in glutaraldehyde cross-linked albumin microspheres (2-5 μm diameter) as a means of retaining steroidal drugs within the synovial joint cavity. Prednisolone, prednisolone succinate, and methotrexate were incorporated into microspheres with drug loading levels of 1.9, 2.6 and 5.0% w/w respectively being obtained. Lewis *et al.*, (1992), reported the incorporation of hydrocortisone into thermal-denatured albumin microspheres (mean diameter 3.1 μm) with a drug loading of 12-14% w/w. *In vivo* studies, using the rat hind-paw model of inflammation to test the efficacy of the drug delivery system, showed enhanced reduction in hind-paw oedema formation after oral administration of drug-incorporated microspheres, when compared to similar concentrations of free hydrocortisone.

PLA microspheres have also been investigated as to their loading of steroids and their potential uses. Beck *et al.*, (1979), proposed the use of progesterone-incorporated d,l-

PLA microspheres (43-61 μm diameter, 22% w/w progesterone) as an injectable, sustained release system for a contraceptive device. Hydrocortisone, as a model water-insoluble drug, has been incorporated into d,l-PLA microspheres (250-350 μm diameter, 12-47% w/w hydrocortisone) with the hydrocortisone being shown to be stable and dispersed within the PLA matrix (Cavalier *et al.*, 1986). Mahato *et al.*, (1992), investigated the potential use of hydrocortisone-incorporated poly (d,l-lactide-co-glycolide) (PLGA) microspheres (mean diameter 1.5 μm , 1-7.5% w/w hydrocortisone) as a possible controlled release system for corticosteroids for intra-articular injection.

The aim of this study was to prepare hydrocortisone-incorporated albumin and PLA microspheres, with a mean diameter $\leq 3 \mu\text{m}$, and to optimize the drug loading levels and drug release profiles *in vitro* of these microspheres for future *in vivo* investigations into their use as a potential drug delivery system for the treatment of chronic inflammation.

4.2 MATERIALS

All chemicals and solvents were purchased from appropriate chemical suppliers as listed in Appendix I. HPLC grade solvents and water were used for MP preparations. All other chemicals were either of Analar or reagent grade. Distilled water was used throughout for the preparation of analyte solutions unless stated otherwise.

4.3 INSTRUMENTATION

4.3.1 ANALYTICAL

HPLC analyses were performed using an analytical system constructed from a number of basic components as outlined in Section 3.3.1.

4.3.2 SUBSIDIARY

A Thermoflow water heater (Conair Churchill Ltd., Uxbridge, UK) with 3 x 50 ml water jacketed cells in series were used for dissolution purposes. The contents of each cell was magnetically stirred at the same rate using a Variomag® Electronicrührer Multipoint HP15 stirrer (Camlab Ltd., Cambridge, England). The cells were covered with aluminium foil to prevent heat loss and water evaporation. A microcentrifuge was used for centrifugation purposes at 13,000 rpm. An Edwards Modulyo freeze-drier (BOC Ltd., Sussex, England) was employed for all freeze-drying processes. All pH

measurements were undertaken using a WPA CD 660 Digital pH meter (WPA Linton, Cambridge, England) appropriately calibrated with ColourKey Buffer Solutions (BDH Ltd.). The following Sartorius electronic balances (Sartorius GMBH, Göttingen, Germany) were employed for accurate weighing purposes; an analytical A200S (4 decimal places) and a research R200D (5 decimal places). A flask shaker (SFI, Stuart Scientific Company Ltd., UK) was employed for gentle mixing purposes.

4.4 METHODS

4.4.1 DIRECT INCORPORATION OF HYDROCORTISONE IN ALBUMIN MICROSPHERES

Albumin microspheres were prepared by the thermal-denaturation method as described in Section 2.4.1.1, with a single modification. 25 mg HC was suspended in 500 μ l BSA solution (25% w/v) and this was then used to form thermal-denatured microspheres. The protein and drug suspension was left to equilibrate for 1 hr prior to the formation of microspheres, since it has been suggested that the higher the degree of binding between the drug and the protein molecules, the greater is the entrapment capacity of the protein microspheres (Oppenheim *et al.*, 1984). In order to ensure maximum binding between drug and albumin is obtained, Yapel (1979), suggested an equilibrium period of 15 to 60 min before dispersing the drug-protein solution in oil. After freeze-drying overnight, the prepared microspheres were stored desiccated at room temperature.

4.4.2 INDIRECT INCORPORATION OF HYDROCORTISONE BY ENZYMATIC CLEAVAGE OF HYDROCORTISONE-21-PHOSPHATE IN ALBUMIN MICROSPHERES

Due to the low solubility of HC in water (0.28 mg ml⁻¹), Lewis *et al.*, (1992), developed a method whereby the freely soluble di-sodium salt of HC, H-21-P, was incorporated into the w/o emulsion during microsphere preparation. H-21-P was subsequently converted back to HC, by enzymatic cleavage of the phosphate group using the enzyme alkaline phosphatase, resulting in a comparatively higher loading of HC being incorporated in albumin microspheres (12.1-14.7% w/w). For this reason, a method based on that developed by Lewis *et al.*, (1992), was employed for the incorporation of HC into albumin microspheres.

Typically 100 mg H-21-P and 5 mg alkaline phosphatase was dissolved in 500 μ l BSA solution (25% w/v, pH 8.8), pre-cooled to $\leq 4^{\circ}\text{C}$ to prevent premature enzyme activity, and added to 30 ml olive oil. A w/o emulsion was then formed by homogenization ($\geq 15\ 000$ rpm) for 2 min. The emulsion was warmed to $35\pm 2^{\circ}\text{C}$ for 30 min to allow enzymatic conversion of H-21-P to HC, before microsphere stabilization by thermal-denaturation as described in Section 2.4.1.2. After freeze-drying overnight, the prepared microspheres were stored desiccated at room temperature.

To ensure alkaline phosphatase was able to convert H-21-P to HC under microsphere preparation conditions, simultaneous controls were run as follows. 5 mg alkaline phosphatase was added to 500 μ l 25% w/v BSA solution (pH 8.8) containing 100 mg H-21-P and subjected to the same conditions as the microsphere mixture; - incubation at 4°C , followed by warming to $35\pm 2^{\circ}\text{C}$ for 1 hr. The protein was removed by precipitation with cold methanol, followed by centrifugation (13,000 rpm, 5 min). The supernatant was diluted appropriately in methanol, with the addition of internal standard (propyl paraben, $40\ \mu\text{g ml}^{-1}$ final concentration), and HC and H-21-P levels assayed by the HPLC method outlined in Chapter 3.

4.4.3 ADSORPTION OF HYDROCORTISONE ONTO EMPTY ALBUMIN MICROSPHERES

The loading of drugs onto empty albumin microspheres has been described in the literature (Benita *et al.*, 1984; Kramer, 1974; Lewis *et al.*, 1992). The method used in this study was based on that of Lewis *et al.*, (1992). Thermal-denatured albumin microspheres were prepared by the method described in Section 2.4.1.1. A saturated solution of hydrocortisone in methanol was prepared in which albumin microspheres were soaked for 1 hr at room temperature, with continuous gentle agitation on a flask shaker. The microspheres were harvested by centrifugation (2,000 rpm, 15 min), washed three times in diethyl ether to remove excess steroid, and freeze-dried overnight. The microspheres were stored desiccated at room temperature.

4.4.4 INCORPORATION OF HYDROCORTISONE IN PLA MICROSPHERES

PLA microspheres were prepared in the same manner as described in Section 2.4.2 with a single modification of the method. This involved the addition of steroid, typically, 100 mg HC, to form a suspension in the organic phase (5 ml dichloromethane). After washing, the PLA microspheres were freeze-dried overnight and stored desiccated at room temperature.

4.4.5 EXTRACTION AND QUANTITATION OF STEROID INCORPORATED IN ALBUMIN MICROSPHERES

The level of steroid incorporated in albumin microspheres was determined by HPLC analysis, the method of which is described in Chapter 3. Two different methods were used to extract the steroid from the microspheres, using both chemical and biological techniques. Many methods have been cited in the literature to extract incorporated drug from albumin microspheres, and include microsphere digestion in 0.5M acetic acid (Gupta *et al.*, 1989d), microsphere digestion in 2N hydrochloric acid-ethanol-methanol mixture (Egbaria & Friedman, 1990), microsphere digestion in 0.4% w/v trypsin solution (Willmott & Harrison, 1988), and the refluxing of microspheres in methanol (Lewis *et al.*, 1992). Two drug extraction methods were compared initially, those of Lewis *et al.*, (1992), and Willmott & Harrison, (1988), and these are outlined below. Subsequently, trypsin degradation was routinely used to extract incorporated steroid from the prepared albumin microspheres.

4.4.5.1 METHANOL REFLUX

Accurately weighed samples (typically 10 mg) of steroid-incorporated albumin microspheres were refluxed in 10 ml aliquots of methanol for 5 hr. After centrifugation (2,000 rpm, 15 min) to remove the microspheres, the supernatants were diluted appropriately in methanol, with the addition of internal standard (propyl paraben, 40 $\mu\text{g ml}^{-1}$ final concentration), and assayed by the HPLC method outlined in Chapter 3. The steroid-incorporation level was expressed as the % gram of drug per gram of microspheres (% w/w).

4.4.5.2 TRYPSIN DEGRADATION

Accurately weighed samples (typically 10 mg) of steroid-incorporated albumin microspheres were digested in 5 ml aliquots of 0.4% w/v trypsin solution at 37°C for 24 hr, after which the mixtures were centrifuged (2,000 rpm, 15 min) to remove large pieces of degraded microspheres. To 1 ml aliquots of the supernatant was added 5 ml ice cold methanol, precipitating any excess protein remaining in the solution. 2 ml aliquots were diluted appropriately in methanol, with the addition of internal standard (propyl paraben, 40 $\mu\text{g ml}^{-1}$ final concentration), and assayed by the HPLC method outlined in Chapter 3. The steroid-incorporation level was expressed as the % gram of drug per gram of microspheres (% w/w).

4.4.6 EXTRACTION AND QUANTITATION OF STEROID INCORPORATED IN PLA MICROSPHERES

The steroid content of PLA microspheres was determined by dissolving accurately weighed samples (typically 10 mg) of prepared microspheres in 5 ml dichloromethane. The PLA was precipitated by the addition of known volumes of excess ethanol and the solutions centrifuged (13,000 rpm, 5 min) to pellet the precipitate. 1 ml aliquots of the supernatants were diluted appropriately in methanol, with the addition of internal standard (propyl paraben, 40 $\mu\text{g ml}^{-1}$ final concentration), and assayed by the HPLC method outlined in Chapter 3. The steroid-incorporation level was expressed as the % gram of drug per gram of microspheres (% w/w).

4.4.7 DETERMINATION OF STEROID RELEASE PROFILES FROM ALBUMIN AND PLA MICROSPHERES

Accurately weighed samples (typically 10 mg) of albumin and PLA microspheres were suspended in 50 ml 0.0067M phosphate buffer pH 7.0 (Appendix II) in water-jacketed cells connected to a thermoflow water heater at 37°C. The microsphere suspensions were continually magnetically stirred at the same rate (300 rpm) using a Variomag® multipoint stirrer. 1 ml aliquots of the dissolution fluid were withdrawn periodically, and an equivalent volume of fresh buffer was added back, maintaining a constant volume of dissolution medium under sink conditions. Dissolution samples were diluted appropriately in methanol and internal standard (propyl paraben, 40 $\mu\text{g ml}^{-1}$ final concentration), was added. The drug content of the samples were analysed by the HPLC method outlined in Chapter 3.

4.4.8 DETERMINATION OF HYDROCORTISONE SOLUBILITY IN VARIOUS SOLVENT SYSTEMS

During the preparation of various different PLA microsphere formulations, the solubility of hydrocortisone in the aqueous and organic phases is an important consideration, with steroid-incorporation levels being directly affected by this parameter. The following method was employed to determine the solubility of hydrocortisone in the desired solvent system.

Each determination of HC solubility was set up in triplicate. Excess HC was added to 1.5 ml solvent in glass Bijou bottles, and the suspension incubated overnight in a shaking water bath at 25°C. Excess HC was removed by filtration through 0.5 μm

pore, teflon, solvent-resistant filters (Millex-SR, Millipore Waters Ltd., UK). 1 ml aliquots of the HC solutions were diluted appropriately in methanol, with the addition of internal standard (propyl paraben, 40 $\mu\text{g mg}^{-1}$ final concentration), and assayed by the HPLC method outlined in Chapter 3.

4.4.9 PARTICLE SIZE ANALYSIS

Particle size was determined by SEM as outlined in Section 2.4.3.

4.4.10 EXAMINATION OF STEROID-INCORPORATED ALBUMIN MICROSPHERES BY TRANSMISSION ELECTRON MICROSCOPY

Steroid-incorporated, thermal-denatured albumin microspheres were suspended in a 1% agar solution, cooled to 4°C and 1 x 1 x 2 mm sections of the agar cut. Chemical fixation of the microspheres involved a primary fixative step of immersion in 2.5% glutaraldehyde for 1 hr, followed by a secondary fixative process of immersion in 1% osmium tetroxide for 1 hr. The samples were dehydrated through a series of 70-100% ethanol and propylene oxide baths, followed by immersion in a 1:1 mix of propylene oxide and resin for 45 min. The fixed samples were embedded in epoxy resin under vacuum for 20 min, followed by polymerization at atmospheric pressure for 24 hr at 60°C. The sample blocks were roughly trimmed to the area containing the microspheres. 70 nm sections were cut and collected onto an electron microscope grid. The sections were stained with uranyl acetate and Reynold's lead citrate before being examined by transmission electron microscopy (TEM), with photomicrographs of sectioned microspheres being taken.

4.5 EXPERIMENTAL

4.5.1 EFFECT OF STEROID:ALBUMIN RATIO ON STEROID INCORPORATION LEVELS AND STEROID RELEASE PROFILES FROM THERMAL-DENATURED ALBUMIN MICROSPHERES

It has been reported in the literature that an increase in the drug:protein concentration ratio increases the drug content of the final delivery device (Oppenheim, 1981; Tomlinson & Burger, 1985). This study was undertaken to locate an optimum drug:protein concentration ratio, at which the drug entrapment was increased without substantially reducing the yield of the final drug delivery system.

The protein concentration in this study was kept constant at 25% w/v BSA. Since 0.5 ml BSA solution is employed in the aqueous phase, the total protein in each batch of microspheres was 125 mg BSA. HC was added to the aqueous phase in the following amounts: 12.5, 25, 50, 100 and 125 mg, giving steroid:protein ratios ranging from 1:10 to 1:1. All formulations of microspheres were prepared in triplicate, with thermal-denaturation at $125\pm 5^\circ\text{C}$ for 30 min.

The total % w/w steroid-incorporation levels, and the HC and H-21-P incorporation levels were determined by HPLC analysis, after enzymatic degradation of samples from each microsphere batch. Drug release profiles for each microsphere batch were determined as described in Section 4.4.7. Microspheres from each batch were examined by SEM, with particle size determinations being carried out as outlined in Section 2.4.3.

4.5.2 EFFECT OF DENATURATION TIME ON STEROID-INCORPORATION LEVELS AND STEROID RELEASE PROFILES FROM THERMAL-DENATURED ALBUMIN MICROSPHERES

The degree of carrier stabilization is known to effect the incorporation level of drugs (Gupta *et al.*, 1989d). The greater the degree of stabilization, the smaller the degree of drug incorporation. This study set out to determine what effect increasing the thermal-denaturation time had on the incorporation of steroid in albumin microspheres using the enzyme conversion method of drug incorporation. A steroid:protein ratio of 1:1.25 was consistently used, and the length of thermal denaturation at $125\pm 5^\circ\text{C}$ was increased from 30 min through to 8 hr. All formulations of microspheres were prepared in triplicate.

The total % w/w steroid-incorporation levels, and the HC and H-21-P incorporation levels were determined by HPLC analysis, after enzymatic degradation of samples from each microsphere batch. Drug release profiles for each microsphere batch were determined as described in Section 4.4.7. Microspheres from each batch were examined by SEM, with particle size determinations being carried out as outlined in Section 2.4.3.

4.5.3 EFFECT OF DENATURATION TEMPERATURE ON STEROID-INCORPORATION LEVELS AND STEROID RELEASE PROFILES FROM THERMAL-DENATURED ALBUMIN MICROSPHERES

The temperature of thermal-denaturation as well as the length of denaturation has also been shown to effect drug incorporation and drug release profiles from albumin microspheres (Gupta *et al.*, 1986b). This study set out to determine the effect of increasing the thermal-denaturation temperature from $100\pm 5^{\circ}\text{C}$ through to $155\pm 5^{\circ}\text{C}$. A steroid:protein ratio of 1:1.25, and a 3 hr thermal-denaturation time were consistently used throughout the series of experiments. All formulations of microspheres were prepared in triplicate.

The total % w/w steroid-incorporation levels, and the HC and H-21-P incorporation levels were determined by HPLC analysis, after enzymatic degradation of samples from each microsphere batch. Drug release profiles for each microsphere batch were determined as described in Section 4.4.7. Microspheres from each batch were examined by SEM, with particle size determinations being carried out as outlined in Section 2.4.3.

4.5.4 EFFECT OF STEROID:POLYMER RATIO ON STEROID-INCORPORATION LEVELS IN PLA MICROSPHERES

The effect of increasing the steroid:polymer ratio on steroid incorporation in PLA microspheres was investigated in this series of experiments. The polymer concentration was kept constant throughout, with the amount of HC being added set at 25, 50 and 100 mg, to give steroid:polymer ratios of 1:10, 1:5 and 1:2.5 respectively. All formulations were prepared in triplicate. The % w/w HC incorporation level for each microsphere batch was determined as outlined in Section 4.4.6. Microspheres from each batch were examined by SEM, with particle size determinations being carried out as outlined in Section 2.4.3.

4.5.5 EFFECT OF STEROID SATURATION OF THE AQUEOUS PHASE ON STEROID-INCORPORATION LEVELS IN PLA MICROSPHERES

Due to low HC incorporation levels ($1.22\pm 1.10\%$ w/w, see Table 4.5) it was thought that HC may be partitioning into the aqueous phase during microsphere formation and washing procedures. The solubility of HC in dichloromethane and 1.5% w/v PVA solution was assayed as outlined in Section 4.4.8. It was determined that HC has a

solubility in 1.5% w/v PVA solution of 1.38 mg ml⁻¹, compared to 0.28 mg ml⁻¹ in water and 0.83 mg ml⁻¹ in DCM, at 25°C (see Table 4.6). Thus, if 75 ml of aqueous phase is used during PLA microsphere preparation, ≥ 100 mg HC may be dissolved in this phase. For this reason, PLA microspheres were prepared using a steroid:polymer ratio of 1:2.5, with a HC saturated 1.5% w/v PVA solution.

4.5.6 EFFECT OF INCREASING STEROID SOLUBILITY IN VARIOUS SOLVENTS ON STEROID-INCORPORATION LEVELS IN PLA MICROSPHERES

Since the solubility of HC in dichloromethane is comparatively low (0.83 mg ml⁻¹), drug loadings exceeding this concentration had to be prepared from a suspension of HC in dichloromethane, resulting in poor drug incorporation levels (1.22±1.10% w/w, see Table 4.5). The use of a co-solvent such as dimethylsulphoxide (DMSO) and tetrahydrofuran (THF) has been reported to increase drug incorporation in PLGA microspheres (Mahato *et al.*, 1992). Using DMSO, the solubility of HC in 100% DMSO and at 1%, 3% and 5% v/v DMSO in dichloromethane was investigated using the method outlined in Section 4.4.8. 5% v/v DMSO in DCM gave rise to a HC solubility of 28.63 mg ml⁻¹, thus this co-solvent system was used to prepare PLA microspheres using a steroid:polymer ratio of 1:2.5.

Higher HC loadings were achieved (4.86±0.39% w/w, see Table 4.7), so the co-solvent system was employed, along with the use of HC saturated 1.5% w/v PVA solutions, to determine whether this would further increase the HC loading of PLA microspheres.

4.5.7 STEROID-RELEASE PROFILES FROM STEROID-INCORPORATED PLA MICROSPHERES

Once a sufficient HC-incorporation level had been achieved using a combination of co-solvent and HC saturation of the aqueous phase techniques, drug release profiles for each microsphere batch were determined as described in Section 4.4.7.

4.6 RESULTS AND DISCUSSION

4.6.1 STEROID-INCORPORATION IN THERMAL-DENATURED ALBUMIN MICROSPHERES

Three methods were initially assessed to incorporate HC into albumin microspheres; direct incorporation by the suspension of HC in the aqueous phase, indirect incorporation of HC by the enzymatic conversion of H-21-P into HC during incubation of the w/o emulsion phase, and thirdly, adsorption of steroid onto the microsphere surface by the soaking of empty microspheres in HC-saturated methanol solutions. In all preparations, albumin microspheres were produced in unagglomerated, free-flowing powders, and examination by SEM revealed spherical particles.

Two methods were assessed to find the most efficient protocol for extracting incorporated steroid from albumin microspheres, firstly by refluxing the microspheres in methanol, and secondly by degrading microspheres with the proteolytic enzyme, trypsin. It was found that trypsin degradation of the albumin microspheres gave rise to slightly higher estimates of steroid-loading levels than did the methanol reflux method. It was proposed that this was probably due the release of internally trapped steroid after matrix degradation with trypsin (see Figure 4.1). No visible signs of degradation of the microspheres after methanol reflux could be seen by SEM examination. Previous studies (Gupta *et al.*, 1986a; 1987a) have advocated the use of two successive proteolytic digestions. However, no difference was obtained in steroid-incorporation measurements by repeatedly degrading the albumin microspheres in this study.

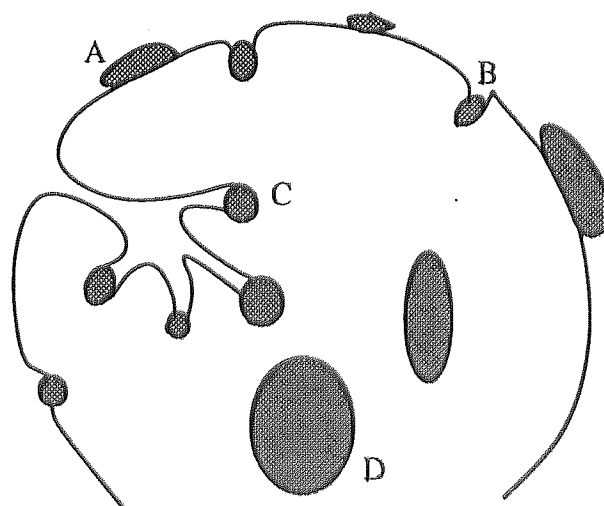


FIGURE 4.1 *A Hypothetical Picture Of An Albumin Microsphere Showing Possible Sites Of Location Of Drug (adapted from Gupta et al., 1986)*

Using the HPLC method developed and outlined in Chapter 3, the total steroid-incorporation levels, as well as the individual incorporation levels of HC and H-21-P, were determined for each different microsphere formulation. HPLC analysis of the microsphere-incorporated steroid showed no alteration in the retention times or symmetrical peaks for HC and H-21-P, as compared to those of standard drug solutions analysed under identical chromatographic conditions. This would indicate that the thermal-denaturation and chemical cross-linking processes were not degrading the steroids during the microsphere manufacturing process, and neither was the enzymatic degradation process used to recover incorporated steroid from the microspheres.

In order to determine the location of steroid within the microspheres, steroid-incorporated albumin microspheres, prepared as described in Section 4.4.2 with thermal denaturation at $125\pm 5^\circ\text{C}$ for 30 min, were examined by TEM both before and after being subjected to dissolution for 30 min at 37°C (Plate 4.1 and 4.2 respectively). Before dissolution it appears that the steroid is incorporated homogeneously within the albumin microsphere. However, after dissolution, there appears to be pockets from where the steroid has been released from the microspheres.

Table 4.1 shows the initial results obtained for the loading of steroid by the three different preparation methods investigated. Direct incorporation of HC in thermal-denatured ($125\pm 5^\circ\text{C}$, 30 min) albumin microspheres, using an initial HC:protein ratio of 1:5, gave rise to steroid-incorporated albumin microspheres with a HC-incorporation level of $1.9\pm 0.6\%$ w/w ($n=3$), and a representative batch manufactured in this way having a geometric mean diameter $\pm \sigma_g$ of $2.43\pm 0.88 \mu\text{m}$. These results are comparable to those of Saunders *et al.*, (1991), who reported a prednisolone incorporation of 1.9% w/w in glutaraldehyde cross-linked albumin microspheres with a mean diameter of 2-5 μm . Since HC is only slightly soluble in water (0.28 mg ml^{-1}), and the aqueous phase volume was 0.5 ml, $\leq 0.56\%$ of the added drug will be in solution, the rest either remaining in suspension or bound to the albumin. It has been proposed by Burgess *et al.*, (1987), that during microsphere formation the BSA will fuse around the suspended drug, with the drug being contained within the microspheres as solid particles. SEM examination of albumin microspheres prepared by this direct method of HC loading, showed smooth surfaced, spherical microspheres with no evidence of drug crystallization on their surface, thus indicating that the steroid was probably incorporated within the albumin matrix.

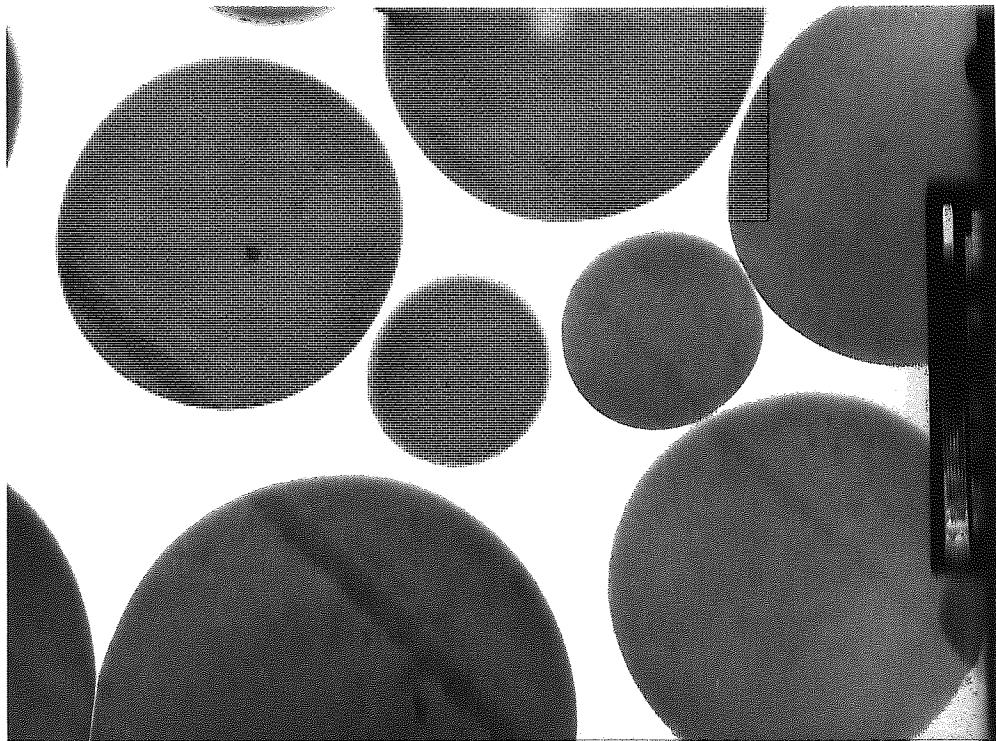


PLATE 4.1 *Transmission Electron Micrograph Of Steroid-Incorporated Albumin Microspheres Prepared By Thermal-Denaturation At 125 ± 5 °C For 30 Minutes*

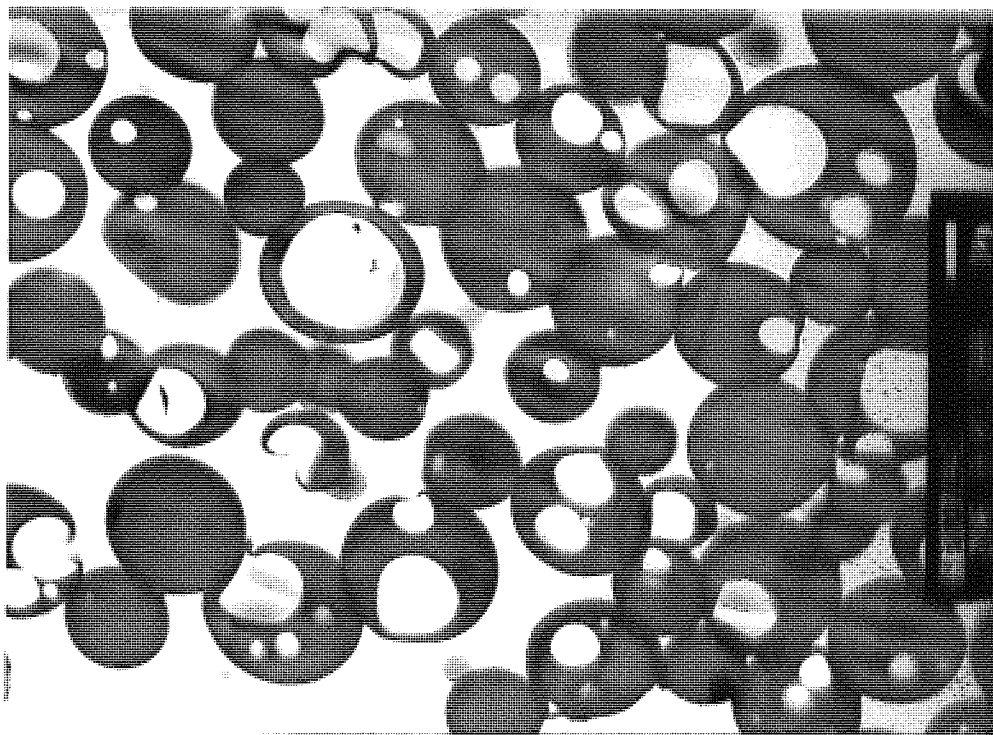


PLATE 4.2 *Transmission Electron Micrograph Of Steroid-Incorporated Albumin Microspheres Prepared By Thermal-Denaturation At 125 ± 5 °C For 30 Minutes And Subjected To 30 Minutes Dissolution At 37 °C*

(contrast to Steroid?)

TABLE 4.1 Comparison Of Three Methods Of Hydrocortisone-Incorporation In Thermal-Denatured ($125 \pm 5^\circ\text{C}$, 30 min) Albumin Microspheres
Each figure represents the mean \pm sd ($n=3$)

METHOD OF STEROID-INCORPORATION	DRUG: ALBUMIN RATIO	TOTAL STEROID-INCORPORATION (% w/w)	H-21-P INCORPORATION (% w/w)	HC INCORPORATION (% w/w)	GEOMETRIC MEAN DIAMETER AND σ_g † (μm)
Direct HC Loading	1:5	1.9 ± 0.6	-	1.9 ± 0.6	2.65 ± 1.18
Indirect HC Loading	1:1.25	30.1 ± 3.0	28.8 ± 3.4	1.4 ± 0.5	3.19 ± 1.45
Adsorption Of HC Onto Empty Microspheres	-	0.34 ± 0.2	-	0.34 ± 0.2	2.43 ± 0.88

†Geometric mean diameter \pm σ_g of representative microsphere batch

Indirect incorporation of HC by enzymatic cleavage of H-21-P gave relatively high total steroid-incorporation levels of $30.1 \pm 3.0\%$ w/w, with a representative batch having a geometric mean diameter \pm σ_g of $3.19 \pm 1.45 \mu\text{m}$. However, HC-incorporation was low, with only 1.4% w/w of the total steroid incorporated being HC. The conversion of H-21-P to HC was shown to occur in 25% w/v BSA solutions under similar conditions used in microsphere preparation, with $\geq 80\%$ of the H-21-P being converted to HC. It is proposed that the low loading of HC may be due to its partition into the oil phase during microsphere manufacture, and not due to the efficiency of enzymatic conversion of H-21-P to HC. These results are in contrast to those of Lewis *et al.*, (1992), who reported HC-incorporation levels of $12.1-14.7\%$ w/w (geometric mean diameter \pm σ_g of $3.1 \pm 1.7 \mu\text{m}$), using the same method and identical experimental conditions. No reference was made as to H-21-P-incorporation levels, so it is assumed all H-21-P was converted to HC.

The adsorption of HC onto the surface of albumin microspheres is a physical process. The degree of adsorption of drug is dependent upon the number of binding groups available on the particle surface (Royer *et al.*, 1983). HC loaded onto empty albumin microspheres in this way gave a very low steroid loading level of $0.34 \pm 0.2\%$ w/w.

This may be due a small number of HC binding sites available on the microsphere surface. It could also be due to the washing of the microspheres in diethyl ether, in which HC is slightly soluble. Using fewer washing steps and smaller volumes of diethyl ether in each washing step, it was possible to raise the HC loading to around 2% w/w. However, SEM examination of these microspheres showed drug crystals deposited on the surface of the microspheres. This will be washed off as soon as the microspheres are suspended in a suitable carrier medium, so it was not considered to be an advantage. Yapel (1979), has reported the loading of drugs onto albumin microspheres using this method is generally low, (<5% of microsphere weight), and must be reserved for highly potent drugs. The results obtained in this study again contrasts with those of Lewis *et al.*, (1992), who reported a HC loading of 6-9% using a similar method of HC adsorption.

With respect to the above results, the method chosen to continue investigating the incorporation of steroid in albumin microspheres was the indirect HC-incorporation method using enzymatic conversion of H-21-P into HC. This method gave a relatively high total steroid loading, and a HC loading not much lower than that of the direct HC-incorporation method.

4.6.1.1 EFFECT OF STEROID:ALBUMIN RATIO ON STEROID-INCORPORATION LEVELS AND STEROID RELEASE FROM THERMAL-DENATURED ALBUMIN MICROSPHERES

The effect of increasing the steroid:albumin ratio in the aqueous phase on steroid-incorporation levels is shown in Table 4.2. This table also shows the effect of increasing the level of steroid incorporation on the geometric mean diameter of the particles. As the initial drug weight was increased during microsphere preparations from 12.5 to 125 mg, there was an increase in the steroid-incorporation level from $7.9 \pm 0.5\%$ w/w to $36.9 \pm 3\%$ w/w. This trend has previously been demonstrated by other groups. Sheu *et al.*, (1986), using 1-Norgestrel, demonstrated that at a constant albumin concentration, an increase in the initial content of the drug in the aqueous phase from 100 μg to 500 μg , resulted in an increase in drug incorporation from 5% w/w to 31% w/w. Similarly, as the steroid content increased, so did the mean particle diameter.

Another way of increasing drug incorporation in albumin microspheres is to reduce the protein concentration. Tomlinson & Burger, (1985), using sodium cromoglycate as a model drug, demonstrated that at a constant drug concentration, a decrease in the

aqueous albumin concentration from 25% w/v to 1% w/v resulted in an increase in drug incorporation from 16% w/w to 83% w/w. However, such overloading of microspheres can result in an undesirable deposition of drug crystals at the carrier surface (Lee *et al.*, 1981), and, moreover, the use of such low concentrations of protein results in poor yields of microspheres. Since the microsphere yields using a 25% w/v albumin solution were in the range of 55-65% (70-80 mg microspheres), it was not considered practical to attempt to improve the steroid-incorporation level by reducing the protein concentration in these studies.

The effect of increasing the initial steroid concentration in the aqueous phase on steroid release from albumin microspheres is shown in Figure 4.2. As the steroid payload increases, so the release of steroid increases, with >98.5% of the incorporated steroid being released within the first 0.5 hr when prepared at a steroid:albumin ratio of 1:1. This trend has also been observed by other workers (Sokoloski & Royer, 1984, Tomlinson *et al.*, 1984b).

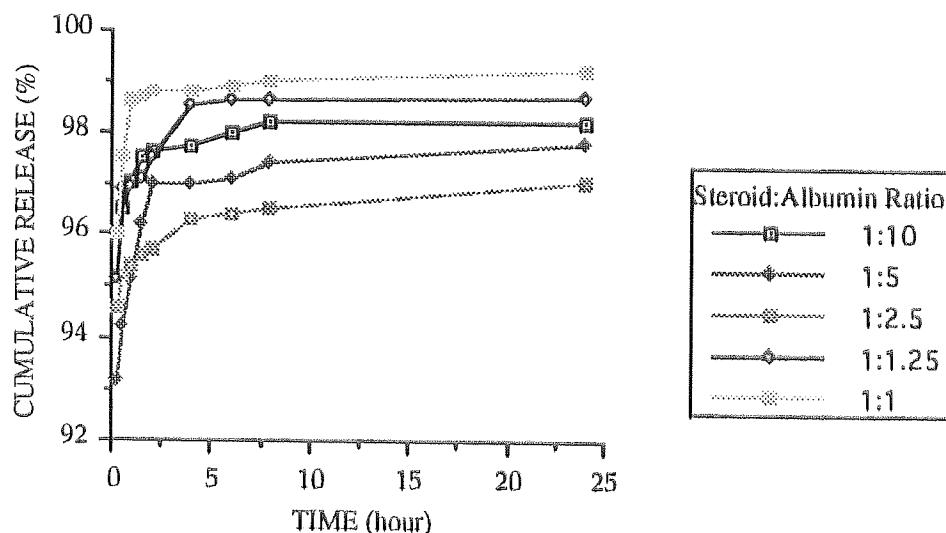
TABLE 4.2 *Effect Of Steroid:Albumin Ratio On Steroid-Incorporation Levels And Particle Size Of Thermal-Denatured (125±5 °C, 30 min) Albumin Microspheres*
Each figure represents the mean±sd (n=3)

STEROID: ALBUMIN RATIO	TOTAL STEROID- INCORPORATION (% w/w)	H-21-P INCORPORATION (% w/w)	HC INCORPORATION (% w/w)	GEOMETRIC MEAN DIAMETER AND σg† (μm)
1:1	36.9±3.0	34.0±5.5	2.9±0.4	3.88±1.74
1:1.25	30.1±3.0	28.8±3.4	1.4±0.5	3.19±1.45
1:2.50	27.4±1.2	25.3±0.9	2.1±0.3	2.18±1.01
1:5	12.6±1.7	14.1±1.4	1.5±0.4	2.39±1.23
1:10	7.9±0.5	5.8±0.4	2.2±0.3	2.07±0.88

†Geometric mean diameter ± σg of representative microsphere batch

FIGURE 4.2 *Release Of Steroid From Thermal-Denatured (125 ± 5 °C, 30 min) Albumin Microspheres Prepared With Varying Steroid:Albumin Ratios. Dissolution In 0.0067M Phosphate Buffer, pH 7.0, 37 °C, Under Sink Conditions.*

Each point represents mean (n=3). Error bars representing standard deviation from the mean have been omitted for clarity. No significant difference was seen in steroid release from each formulation.



4.6.1.2 EFFECT OF THERMAL-DENATURATION TIME ON STEROID-INCORPORATION LEVELS AND STEROID RELEASE FROM THERMAL-DENATURED ALBUMIN MICROSPHERES

The time for which heat is maintained at a given temperature during the process of carrier stabilization has been shown to influence the level of drug incorporation in albumin microspheres (Gupta *et al.*, 1988b), with the longer the length of stabilization, the smaller the drug incorporation level. The influence of length of stabilization was investigated in this study, with thermal-denaturation times increasing from 0.5 hr up to 8 hr. The results, shown in Table 4.3, show that as the length of stabilization increases from 0.5 hr to 8 hr, so the level of drug incorporation decreases from $30.1 \pm 3.0\%$ w/w to $6.3 \pm 0.8\%$ w/w, along with a decrease in particle diameter. These trends may both be explained by an increase in the compactness of the protein matrix, with there being less space for entrapped drug to lie within the albumin matrix after more extensive denaturation. The degree of denaturation has also been shown to affect the release rate of entrapped drug from the microsphere carrier. According to most workers, an increase in carrier stabilization time increases the tortuosity of the particle matrix, leading to an overall decrease in the rate of release of drug (Lee *et al.*, 1981, Morimoto *et al.*, 1985). The *in vitro* steroid release profiles obtained for steroid-incorporated

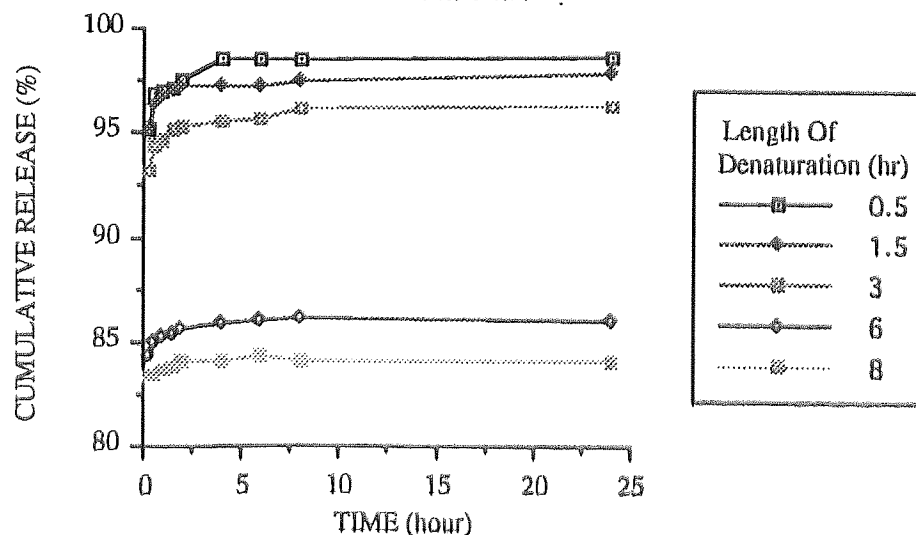
albumin microspheres with increasing lengths of thermal denaturation during preparation are shown in Figure 4.3.

TABLE 4.3 *Effect Of Increasing Thermal-Denaturation ($125\pm 5^\circ\text{C}$) Time On Steroid-Incorporation And Particle Size In Albumin Microspheres*
Each figure represents the mean \pm sd ($n=3$)

LENGTH OF THERMAL-DENATURATION (hr)	TOTAL STEROID-INCORPORATION (% w/w)	H-21-P INCORPORATION (% w/w)	HC INCORPORATION (% w/w)	GEOMETRIC MEAN DIAMETER AND σ_g † (μm)
0.5	30.1 \pm 3.0	28.8 \pm 3.4	1.4 \pm 0.5	3.19 \pm 1.45
1.5	25.9 \pm 0.8	23.4 \pm 0.8	1.8 \pm 0.5	2.36 \pm 1.19
3	15.5 \pm 1.0	13.6 \pm 0.8	1.9 \pm 0.2	2.49 \pm 1.32
6	8.3 \pm 0.9	7.4 \pm 0.8	0.9 \pm 0.1	2.39 \pm 1.02
8	6.3 \pm 0.8	5.7 \pm 0.6	0.6 \pm 0.2	2.14 \pm 0.98

†Geometric mean diameter and σ_g of representative microsphere batch

FIGURE 4.3 *Release Of Steroid From Thermal-Denatured Albumin Microspheres Prepared With Varying Time Lengths Of Thermal Denaturation ($125\pm 5^\circ\text{C}$) Using A Steroid:Albumin Ratio Of 1:1.25. Dissolution In 0.0067M Phosphate Buffer, pH 7.0, 37°C , Under Sink Conditions. Each point represents mean ($n=3$). Error bars representing standard deviation from the mean have been omitted for clarity. No significant difference was seen in steroid release from each formulation between 0.5 and 3 hr and between 6 and 8 hr.*



4.6.1.3 EFFECT OF DENATURATION TEMPERATURE ON STEROID-INCORPORATION LEVELS AND STEROID RELEASE FROM THERMAL-DENATURED ALBUMIN MICROSPHERES

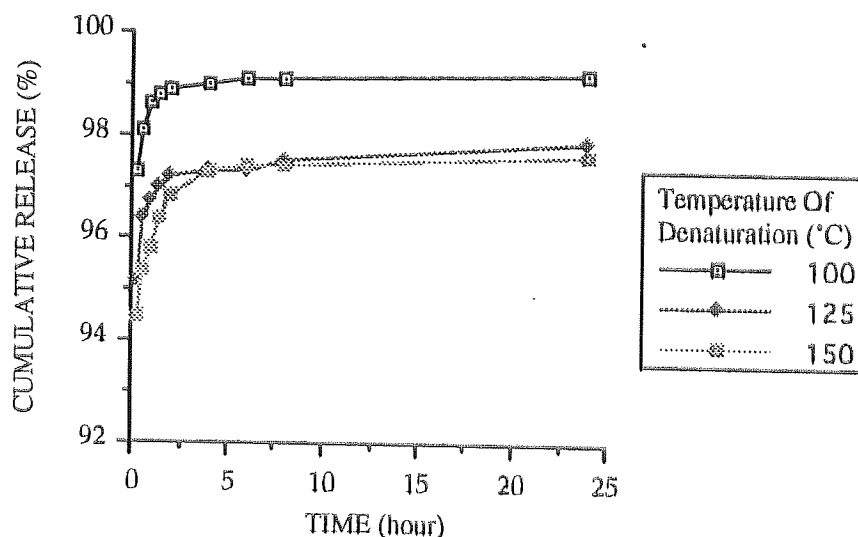
The effect of increasing the temperature of stabilization on steroid-incorporation levels and geometric mean particle size is shown in Table 4.4. The length of denaturation was kept constant at 1.5 hr, since microspheres prepared with this length of stabilization were shown to have relatively high steroid-incorporation levels ($25.9 \pm 0.8\%$ w/w), with geometric mean diameters around $2.4 \mu\text{m}$ (Table 4.3). Thermal-denaturation at 100°C gave rise to slightly larger microspheres and a slightly higher steroid-incorporation level than obtained in preparations with higher denaturation temperatures of 125°C and 150°C . Trends reported in the literature (Gupta *et al*, 1987a; 1988b) show a decrease in drug incorporation levels as the denaturation temperature is increased. However, little difference was seen in steroid-incorporation levels and microsphere size at the two higher temperatures. It is thought that the microspheres formed at the higher temperatures have a more dense matrix than those formed at the lower temperature, excluding drug from incorporation within the matrix. It has also been shown that the temperature of heat stabilization may be used to control the release of the entrapped drug from albumin microspheres (Gupta *et al*, 1986a; 1988d). However, the steroid release profiles obtained from the steroid-incorporated albumin microspheres prepared at 100°C , 125°C and 150°C (Figure 4.4) show little difference in their steroid release patterns, with $>96\%$ steroid being released within the first two hours of dissolution.

TABLE 4.4 *Effect Of Increasing Thermal-Denaturation Temperature On Steroid-Incorporation And Particle Size In Albumin Microspheres*
Each figure represents the mean \pm sd ($n=3$)

THERMAL-DENATURATION TEMPERATURE ($\pm 5^\circ\text{C}$)	TOTAL STEROID-INCORPORATION (% w/w)	H-21-P INCORPORATION (% w/w)	HC INCORPORATION (% w/w)	GEOMETRIC MEAN DIAMETER AND σ_g^\dagger (μm)
100	31.1 ± 2.8	29.4 ± 2.4	1.7 ± 2.8	3.04 ± 2.56
125	25.9 ± 0.8	23.4 ± 0.8	1.8 ± 0.5	2.36 ± 1.19
150	27.9 ± 1.4	25.8 ± 0.3	2.1 ± 1.4	2.43 ± 1.75

† Geometric mean diameter $\pm \sigma_g$ of representative microsphere batch

FIGURE 4.4 *Release Of Steroid From Thermal-Denatured (1.5 hr) Albumin Microspheres Prepared With Varying Temperatures Of Thermal Denaturation. Dissolution In 0.0067M Phosphate Buffer, pH 7.0, 37 °C, Under Sink Conditions. Each point represents mean (n=3). Error bars representing standard deviation from the mean have been omitted for clarity. No significant difference was seen in steroid release from each formulation.*



4.6.2 HYDROCORTISONE-INCORPORATION IN PLA MICROSPHERES

Hydrocortisone-incorporated PLA microspheres were prepared by an adaptation of the solvent evaporation method originally developed by Beck *et al.*, (1979). SEM examination showed all microsphere preparations gave rise to spherical particles with geometric mean diameters $\leq 3 \mu\text{m}$. HC-incorporation levels were accurately quantified by HPLC analysis as described in Chapter 3, after PLA microsphere samples were dissolved in dichloromethane releasing all incorporated HC.

4.6.2.1 EFFECT OF HYDROCORTISONE:POLYMER RATIO ON HYDROCORTISONE-INCORPORATION LEVELS IN PLA MICROSPHERES

The effect of increasing the steroid:polymer ratio on HC-incorporation levels was investigated with the results shown in Table 4.5. Relatively low HC-incorporation levels of $1.22 \pm 1.10\%$ w/w were achieved using a high steroid:polymer ratio of 1:2.5. It was proposed that the low HC-incorporation levels achieved was probably due to partitioning of HC into the external aqueous phase (composed of 1.5% w/v PVA solution) during microsphere preparation. Although HC has a relatively low water solubility (0.28 mg ml^{-1}), the addition of 1.5% w/v PVA increases the solubility of HC

to 1.38 mg ml⁻¹ (Table 4.6). Therefore, 75 ml of 1.5% w/v PVA in the external phase was able to dissolve 100 mg HC used in the 1:2.5 steroid:polymer ratio formulation. Since the efficiency of drug incorporation depends upon the degree of drug partitioning into the aqueous phase, a HC saturated 1.5% w/v PVA solution was used as the external phase in an attempt to reduce HC partitioning. This gave rise to a HC incorporation of 4.86±0.39% w/w (Table 4.7). However, SEM examination of the microspheres revealed drug crystals on the surface of the PLA microspheres. Their presence was thought to result from their inability to dissolve in the HC-saturated phase during microsphere formation, and in water during the washing procedures.

TABLE 4.5 *Effect Of Hydrocortisone:Polymer Ratio On Hydrocortisone-Incorporation And Particle Size In PLA Microspheres*
Each figure represents the mean±sd (n=3)

HYDROCORTISONE:POLYMER RATIO	HC INCORPORATION (%w/w)	GEOMETRIC MEAN DIAMETER AND σg† (μm)
1:2.5	1.22±1.10	2.45±1.45
1:5	0.10±0.07	2.65±1.54
1:10	0.08±0.05	2.24±1.07

†Geometric mean and σg of representative microsphere batch

4.6.2.2 USE OF A CO-SOLVENT SYSTEM TO INCREASE HYDROCORTISONE-INCORPORATION LEVELS IN PLA MICROSPHERES

The use of small amounts of DMSO as a co-solvent along with DCM in the internal phase has been suggested to increase the solubility of hydrocortisone, and consequently enhance the loading of steroid in PLGA microspheres (Mahato *et al.*, 1992). A series of solubility studies for HC in dichloromethane, DMSO and 1%, 3%, and 5% v/v DMSO in dichloromethane solutions at 25° C were carried out, with the results shown in Table 4.6. 5% DMSO in dichloromethane was found to enhance HC solubility >34 times that in dichloromethane alone, and this co-solvent system was subsequently employed in the internal phase during HC-incorporated PLA microsphere preparation. By using the co-solvent internal phase, HC-incorporation could be enhanced to 4.99±0.66% w/w (Table 4.7). In addition, use of the co-solvent system along with the use of a HC-saturated external phase gave rise to HC-incorporation levels of

10.6±1.18% w/w. However, as described previously, examination of the particles by SEM showed the formation of drug crystals on the microsphere surface.

TABLE 4.6 *Determination Of Hydrocortisone Solubility At 25 °C In Various Aqueous And Organic Solvent Systems*

SOLVENT SYSTEM	HYDROCORTISONE SOLUBILITY AT 25°C (mg ml ⁻¹)
Water	0.28
1.5% w/v PVA	1.38
Dichloromethane	0.83
DMSO	110.39
1% v/v DMSO in Dichloromethane	4.72
3% v/v DMSO in Dichloromethane	7.98
5% v/v DMSO in Dichloromethane	28.63

TABLE 4.7 *The Effect Of Hydrocortisone Saturation Of The External Phase And The Use Of A Co-Solvent System In The Internal Phase On Hydrocortisone Incorporation In PLA Microspheres*
Each figure represents the mean±sd (n=3)

INTERNAL PHASE	HYDROCORTISONE SATURATION OF EXTERNAL PHASE	HYDROCORTISONE INCORPORATION (% w/w)	GEOMETRIC MEAN DIAMETER AND σg† (µm)
Dichloromethane	-	1.22±1.10	2.45±1.45
Dichloromethane	+	4.86±0.39	2.64±1.53
5% v/v DMSO In Dichloromethane	-	4.99±0.66	2.51±1.11
5% v/v DMSO In Dichloromethane	+	10.60±1.18	2.73±1.74

†Geometric mean and σg of representative microsphere batch

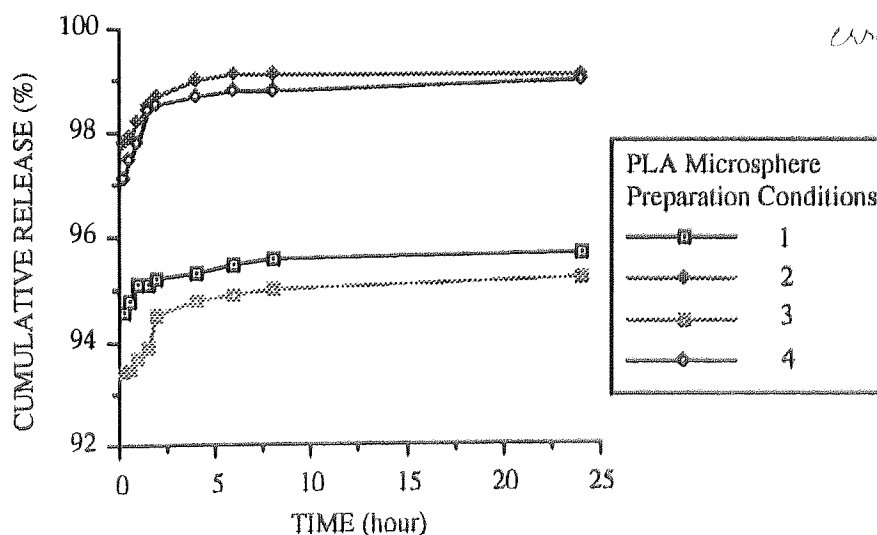
4.6.3 STEROID RELEASE PROFILES FROM PLA MICROSPHERES

The release of HC from PLA microspheres is shown in Figure 4.5. For HC-incorporated formulations prepared using a HC-saturated external phase, >98% of the drug was released within the first 2 hours of dissolution. This initial burst effect was probably due to the drug crystals seen on the surface of the microspheres after preparation by SEM examination. PLA microsphere formulations which were not prepared with drug-saturated external phases also showed high initial burst effects, with greater than >95% of the HC being released within the first 2 hours of dissolution. In both cases, the initial burst effect is followed by a slower release phase over 24 hours. The time length of dissolution was not continued beyond this point since the remaining drug was such a small percentage of the amount incorporated. Mahato *et al.*, (1991), using slightly smaller PLA microspheres (1-2 μm) with much lower drug loadings or 0.74% w/w, reported a burst effect of 20% in the first 3 hours of dissolution in PBS at 37°C, followed by >75% HC released after 24 hr.

FIGURE 4.5 *Release Of Steroid From PLA Microspheres. Dissolution In 0.0067M Phosphate Buffer, pH 7.0, 37°C, Under Sink Conditions.*

Each point represents mean (n=3). Error bars representing standard deviation from the mean have been omitted for clarity

1 - No saturation of the external phase, 2 - HC Saturation of the external phase, 3 - 5% DMSO in the internal phase, no saturation of the external phase, 4 - 5% DMSO in the internal phase, HC saturation of the external phase.



Many factors influence the rate of release of drugs from PLA microspheres including polymer molecular weight, with higher polymer Mwts giving rise to faster drug release rates; particle size, with an increase in the drug release rate as the particle size decreases, as a result of the increase in surface area; and drug incorporation levels, with an increase in the drug release rate as the drug loading increases.

From the experiments carried out in these initial PLA microsphere studies, it is apparent that HC incorporation into microspheres of the required size for oral administration gives rise to relatively low drug loading levels and biphasic drug release profiles, with the HC being released too quickly for these formulations to be viable for *in vivo* experiments. Alternative formulations need to be investigated, with a view to slowing down the release rate of the incorporated drug. The use of more water insoluble hydrocortisone esters offers one possibility. Another possibility would be to incorporate a more potent drug for which relatively low loading levels would provide pharmacologically effective drug doses *in vivo*, alleviating the need to attain higher drug incorporation levels.

4.7 SUMMARY

Hydrocortisone was incorporated in albumin microspheres using three different methods, namely direct incorporation, indirect incorporation *via* conversion of H-21-P to HC, and soaking of empty microspheres in saturated HC solutions. Direct incorporation of HC and adsorption of HC onto the surface of empty albumin microspheres gave rise to relatively low HC incorporation levels of 1.9% w/w and 0.34% w/w respectively. Indirect loading of HC *via* enzymatic conversion of H-21-P to HC gave rise to relatively high steroid incorporation levels of 30.1% w/w. However, only 1.4% w/w of the steroid was HC, the remaining 28.8% being H-21-P. Since high levels of steroid was incorporated in albumin microspheres by this method, it was decided to continue with this preparation technique.

The release of steroid from albumin microspheres was biphasic, with high initial burst phases of >84% of the incorporated drug after 2 hours, despite increasing the length of thermal stabilization. Little effect on slowing down the rate of drug release was achieved by increasing the steroid:albumin ratio and the temperature of thermal stabilization.

HC has been incorporated into PLA microspheres prepared by the solvent evaporation method of microsphere preparation. Initially relatively low HC incorporation levels

were achieved of 1.22% w/w. An increase in the level of drug incorporation was achieved by saturating the external 1.5% w/v PVA aqueous phase with HC, with drug incorporation levels rising to 4.86% w/w. A further increase in drug loading to 10.6% w/w HC was made by using a co-solvent internal phase composed of 5% DMSO in dichloromethane. However, in the latter two formulations, drug crystals were seen on the microsphere surface. HC release from PLA microspheres showed high initial burst phases, with >94% of the HC being released after the first two hours of dissolution.

Although steroid was incorporated in albumin microspheres in relatively high levels (up to 36.9% w/w) their utility as drug delivery systems *in vivo* are severely limited by premature release of the steroid in the solutions used to administer the microspheres.

CHAPTER 5

TISSUE DISTRIBUTION OF ALBUMIN MICROSPHERES FOLLOWING ORAL AND INTRAVENOUS ADMINISTRATION TO RATS INDUCED WITH AN EXPERIMENTAL MODEL OF INFLAMMATION

5. TISSUE DISTRIBUTION OF ALBUMIN MICROSPHERES
FOLLOWING ORAL AND INTRAVENOUS ADMINISTRATION TO
RATS INDUCED WITH AN EXPERIMENTAL MODEL OF
INFLAMMATION

5.1 INTRODUCTION TO THE TARGETING OF MICROSPHERES TO SITES OF
INFLAMMATION

The heterogenic clinical presentation of symptoms by patients suffering with RA hampers the diagnosis and treatment of this debilitating disease. Initial treatment is concerned with alleviating patient pain and discomfort, but as the disease progresses, and becomes more destructive, DMARDs, corticosteroids, and cytotoxic agents are employed in an attempt to retard joint destruction. However, these drugs all cause adverse side-effects, reducing their beneficial effects and limiting their use. Drug delivery systems have been developed in an attempt to relieve these unwanted side-effects. These systems may take two forms for treating inflammation; sustained release preparations, where the delivery device is injected or implanted locally, at the site of inflammation, and targeted drug delivery systems, which may be administered parenterally or intravenously, and reach the desired site of inflammation where they exert their action.

Ratcliffe *et al.*, (1984), investigated the biocompatibility of biodegradable polymer microspheres, including those made from PLA, polybutylcyanoacrylate (PBCA), gelatin, and albumin (1-10 μm diameter), in order to evaluate their suitability as injectable, sustained drug delivery systems. After their intra-articular injection into rabbit knee joints, PLA, PBCA and gelatin microspheres were found to cause joint inflammation, whereas albumin microspheres were well tolerated by the synovial tissues. In a later study, again using arthritic knee joints of rabbits, Ratcliffe *et al.*, (1987), reported the clearance of radioactivity from the arthritic joint after i.a. injection of [^{131}I]-albumin microspheres was slow, 50% of the dose remaining after 3 days, compared to that of free Na^{131}I solution (14% of the dose remaining after 30 minutes). It was thus proposed that albumin microspheres may provide a means of sustaining drug release within the diseased synovium, and could also reduce the rate of drug clearance from the joint. Liposome-entrapped radiolabelled methotrexate, injected into arthritic rabbit joints, has been shown to be retained within the joint much longer than free methotrexate (Foong & Green, 1988). 24 hr after i.a. injection of the free drug, less than 0.6% was recovered from the injected joint, compared with 45% recovery from liposomal methotrexate. However, although these studies advocate the possible

use of sustained delivery systems for patients presenting RA in one or a few joints, this type of treatment is unsuitable for patients with many disease affected small joints.

Non-parenteral and intravenously administered drug delivery systems have been investigated as a means of delivering microsphere-entrapped drug to sites of inflammation throughout the body. Tissue distribution studies of intravenously (i.v.) administered microspheres have shown their presence at various sites of inflammation. Illum *et al.*, (1989), showed the accumulation of [¹³¹I]-labelled polystyrene microspheres (80 nm mean diameter) at the site of carrageenan-induced chronic inflammation in the rabbit left flank, 24 hr after i.v. injection. Particles coated with Poloxamine 908 accumulated at the site of inflammation to a greater extent than non-coated microspheres ($12.5 \pm 2.2\%$ and $0.95 \pm 0.25\%$ of the original dose respectively). Alpar *et al.*, (1989), investigated the distribution of fluorescent latex microspheres (1.1 μm mean diameter) to rat subcutaneous air-pouch models of inflammation 4 hr after i.v. injection into the heart, and 24 hr after oral administration by gavage needle. Microspheres administered orally were found in the tissues and fluid exudates of inflammatory air-pouches in rats, as were microspheres injected directly into the circulation. Further work by the same group (Lewis *et al.*, 1992), using carrageenan-induced hind-paw inflammation in the rat as an inflammatory model, reported albumin microspheres (3.1 μm mean diameter) were able to reach the site of local inflammation from the circulation 24 hr after oral administration by gavage needle. However, no quantitative study of the accumulation of microspheres at the site of inflammation was made in both of the investigations discussed above.

The aim of this study was to attempt to quantify the accumulation of albumin microspheres at the site of inflammation after i.v. injection and oral administration by gavage needle. The coating of albumin microspheres with Poloxamine 908, which reduces the negative surface charge of the particles, is also investigated as to the effect this has on the subsequent tissue distribution of albumin microspheres. Varying degrees of inflammation were induced in an animal model of inflammation and the consequence of this, if any, on microsphere tissue distribution was determined. The animal model of inflammation chosen for this study was that of the rat subcutaneous air-pouch for the reasons explained in the following sections.

5.1.1 THE SUBCUTANEOUS AIR-POUCH MODEL

The subcutaneous air-pouch, also known as the air-pouch granuloma, or granuloma pouch technique, was first described as a potential model system for studying inflammation by Selye, (1953a). The basis of the technique is the subcutaneous (s.c.) injection of sterile air, in rats or mice, to produce a cavity into which a variety of inflammatory and chemotactic stimuli may be introduced. It was subsequently demonstrated that the s.c. injection of air promoted the formation of a cellular lining in the cavity which had many features similar to those of the synovial membrane (Edwards *et al.*, 1981).

Acute inflammatory responses have been initiated in the air-pouch by a variety of inflammatory stimuli including carrageenan (Nakagawa *et al.*, 1983), zymosan (Konno & Tsurifuji, 1983), crystals of monosodium urate (Brooks *et al.*, 1987) and calcium pyrophosphate (Sin *et al.*, 1984a), croton oil (Selye, 1953b), heat-aggregated gamma globulin (Kowanko *et al.*, 1986), and heat-killed mycobacteria (Kowanko *et al.*, 1986). The various aspects of the inflammatory process which have been measured in response to these stimuli include PMNL and mononuclear leukocyte (MNL) accumulation, oedema formation, increase in pouch weight or cellularity, mediator concentration, changes in acute phase proteins, and the effect of anti-inflammatory drugs on these responses. To a large extent, the same inflammatory mediators which have been used for acute inflammation studies have been utilized for chronic inflammation investigations, with the exception that the induced inflammation has been allowed to develop over a longer time period.

The major advantage of using the air-pouch as an experimental model of synovium, as opposed to the knee joint which contains true synovium, lies in its ease of formation and subsequent manipulation. The technical difficulties of introducing inflammatory stimuli and collecting inflammatory exudates from the rat knee joint, without causing physical damage leading to inflammation, are overcome with the air-pouch model. An added advantage is the relatively large volumes of exudate which may be collected from the pouch over a period of time, as opposed to the one off exudate collection from the rat knee joint of approximately 100 μ l. The advantage of using the rat subcutaneous air-pouch model over other more extensively used animal cavity models, such as the rat pleural space and the rabbit peritoneal cavity, lies in the fact that there are no internal organs in the air-pouch cavity which may be punctured or damaged as a result of injections into the cavity.

The obvious disadvantage of the air-pouch model is that it is an animal model and not a human joint. The cavity does not contain cartilage and it is not weight bearing. On balance, the rat subcutaneous air-pouch model is useful to investigate both acute and chronic inflammation, particularly when studying the biochemical and pharmacological aspects of inflammation.

5.1.2 PRINCIPLE USES OF THE SUBCUTANEOUS AIR-POUCH MODEL

The subcutaneous air-pouch has been used primarily for the study of various aspects of the inflammatory process, although it has also been used in pharmacokinetic studies, cell chemotaxis studies and for investigating aspects of tumour induction and growth.

The rat subcutaneous air-pouch provides an ideal system for studying both the appearance into a tissue space of systemically administered drugs (Tsurufugi *et al.*, 1978), and the metabolism of drugs injected directly into the air-pouch (Joyce *et al.*, 1986).

The air-pouch has also been utilised as a novel method for studying both the mechanism of cartilage degradation *in vivo* (Sin *et al.*, 1984b), and the effect of various therapeutic agents on the degradation process (Sedgwick *et al.*, 1984).

A further use of the air-pouch model of inflammation is for chemotaxis studies. The inflammation induced in the air-pouch by a variety of irritants is characterized by vigorous leukocyte infiltration. The chemotactic activity of the exudate may be correlated with leukocyte infiltration (Kurihara *et al.*, 1983), and this used as an assay system to test the ability of drugs to inhibit cell infiltration, and to look at the underlying mechanisms which give the drugs these properties (Kurihara *et al.*, 1984). The air-pouch has been used by Hyde, (1990), to investigate the possibility of targeting microspheres to the site of inflammation. Microspheres were phagocytosed by leukocytes *in vitro*, reintroduced *in vivo* by i.v. injection, and their infiltration into the air-pouch in response to the chemoattractant N-formyl-L-methionyl-L-leucyl-L-phenylalanine assessed.

Finally, the air-pouch has been used for investigating tumour induction and growth. An inherent property of the air-pouch lining is that it contains many rapidly dividing cells and this has been exploited to provide assay systems of mutagenicity (Maier, 1980) and carcinogenicity (Zbinden & Maier, 1983).

5.2 MATERIALS

All chemicals were of Analar or reagent grade and were purchased from appropriate suppliers as listed in Appendix I. [¹²⁵I]-BSA was purchased from NEN Research Products, Dupont Ltd., Stevenage, UK. All solutions and suspensions were made with dH₂O unless otherwise stated.

5.2.1 ANIMALS

Adult male Wistar rats (150-200 g) were purchased from Banting and Kingman, Hull, UK. Animals were housed in the University animal house at 20°C and maintained on Rat and Mouse Breeding Diet (Pilsbury's Limited, Birmingham, U.K.) with water *ad libitum*.

5.2.2 SUBSIDIARY

Boyle's apparatus was used to deliver gases for anaesthetic purposes and a Compugamma Universal Gamma Counter (LKB 1282, Wallac, London, England) was employed to measure [¹²⁵I] radioactivity levels. The following Sartorius electronic balances (Sartorius GMBH, Göttingen, Germany) were employed for accurate weighing purposes; an analytical A200S (4 decimal places) and a research R200D (5 decimal places).

5.3 METHODS

5.3.1 ANAESTHESIA

Halothane (RMB Animal Health Ltd, Dagenham, U.K.) anaesthesia was induced in experimental animals prior to air-pouch formation and administration of antigenic stimuli as follows. The animals were placed in an air chamber connected to Boyle's apparatus. Oxygen and nitrous oxide were mixed in a 1:1 ratio and delivered at a flow rate of 500 cc min⁻¹ through a calibrated vapouriser on the Boyle's apparatus into the air chamber. To initiate anaesthesia, halothane was initially delivered at 4-5% through the Boyle's apparatus. This was subsequently reduced to 1-1.5% for maintenance during surgery or injection, after which the animals were left to recover in normal atmospheric conditions.

A different anaesthetic technique to halothane was used for microsphere tissue distribution studies. Halothane anaesthesia is reported to cause a progressive fall in the systemic blood pressure (Green, 1982) and, as such, was thought to be inappropriate for tissue distribution studies. Instead, the anaesthetic agent used for distribution experiments was fentanyl-fluanisone (Hypnorm, Janssen Pharmaceuticals Ltd., Wantage, Oxon, UK), which was given intra-muscularly (i.m.) into the thigh muscle at a concentration of 0.3 ml kg⁻¹. This dose has been reported to have little effect on cardiac output or blood pressure (Green, 1982), and has a duration of effect lasting for 20-30 minutes.

5.3.2 FORMATION OF 6-DAY OLD SUBCUTANEOUS AIR-POUCHES

The method used for the subcutaneous air-pouch procedure was based on that of Sedgwick *et al.*, (1983). On day 1, male Wistar rats (150-200 g) were anaesthetized with halothane as outlined in Section 5.3.1 and the dorsal and nuchal regions shaved. The shaved area was swabbed with 70% alcohol and 25-30 ml of sterile air was injected s.c. just behind the scapulae using a 25-gauge needle. The air was sterilized by passing it through a 0.2 µm nitrocellulose filter (Millex filter unit, Millipore Waters Ltd, UK) placed between the syringe and needle. The initial injection of 3-4 ml raised a small air-bleb which was manipulated between the thumb and forefinger to produce a regular ellipse as the remainder of the air was injected. The air-pouch formed by this method was a well-defined structure which could readily be dissected away from the underlying tissues. To maintain an open air-pouch a further 10 ml of sterile air was injected on day 3. The air-pouches were routinely used on day 6 after air-pouch formation, which is the time established for the development of an organised cellular lining and blood supply (Sedgwick *et al.*, 1983), and the reported time for full expression of the inflammatory response when challenged with antigen (Sin *et al.*, 1984c).

Handling of the animals after development of the air-pouch was difficult due to the location of the pouch and it was thought inappropriate to scruff the animals in this condition. The animals, where possible therefore, were lightly anaesthetized with halothane before handling during further experimental procedures.

5.3.2.1 FORMATION OF ACUTE INFLAMMATION IN 6-DAY OLD SUBCUTANEOUS AIR-POUCHES

Male Wistar rats with formed 6-day old air-pouches were anaesthetized with halothane. 2 ml 2% w/v sterile carrageenan in physiological saline solution was introduced into the air-pouch with a sterile needle and syringe. The irritant was gently massaged around the air-pouch to ensure even distribution of the viscous solution. Inflammation was routinely left to develop for 24 hr before the animals were used in experimental studies.

5.3.2.2 FORMATION OF CHRONIC INFLAMMATION IN 6-DAY OLD SUBCUTANEOUS AIR-POUCHES

Air-pouches induced with chronic inflammation were formed as in Section 5.3.2.1 with a single modification to the procedure. Inflammation was left to develop routinely over a 72 hr period before the animals were used in experimental studies.

5.3.3 PREPARATION OF RADIOLABELLED ALBUMIN MICROSPHERES

[¹²⁵I]-labelled albumin microspheres were prepared as described in Section 2.4.1.3. Prepared batches were pooled together, and had a geometric mean diameter of $2.67 \pm 1.28 \mu\text{m}$. These microsphere preparations were employed for all tissue distribution experiments.

5.3.4 PREPARATION OF SURFACE MODIFIED RADIOLABELLED ALBUMIN MICROSPHERES

Surface modification of radiolabelled albumin microspheres with Poloxamine 908 was carried out as described in Section 2.4.4.

5.4 EXPERIMENTAL

5.4.1 CHARACTERIZATION OF ACUTE AND CHRONIC INFLAMMATION INDUCED IN SUBCUTANEOUS AIR-POUCHES

Carrageenan-induced subcutaneous air-pouch models of inflammation have been shown to be able to cover not only acute but also chronic stages of the inflammatory process (Fukuhara & Tsurufuji, 1969). The optimum time period for the development of acute and chronic inflammation in the air-pouch, after the introduction of carrageenan

in these studies, was determined, along with the reproducibility of the degree of inflammation. The acute and chronic inflammation induced in the air-pouch tissues was characterized in terms of fluid accumulation, the total number of cells infiltrating into the pouch fluid, along with the type of infiltrating cells and their relative numbers. The increase in the lining tissue of the pouch and its cellular formation was monitored by histological examination.

In all experiments, two sets of controls were used. Firstly, air-bleb control tissues were used to compare tissues in their normal state, as opposed to the air-pouch state. The air-bleb was formed by injecting sterile air into the dorsal region of the rat and excising this tissue immediately prior to use. Secondly, 6-day old air-pouch tissues without carrageenan challenge were used to compare non-inflamed pouch tissues with those subjected to irritant stimuli.

5.4.1.1 STUDY OF ACUTE INFLAMMATION IN THE RAT SUBCUTANEOUS AIR-POUCH MODEL

Acute inflammation was induced in 5 sets of 3 Wistar rats with 6-day old subcutaneous air-pouches, (formed as outlined in Section 5.3.2.1), by the injection of 2 ml 2% w/v sterile carrageenan solution. Groups of animals were sacrificed by CO₂ asphyxiation at 2, 4, 6, 8 and 24 hr after carrageenan challenge. Each pouch was opened, the total fluid accumulated collected and its volume measured. The initial carrageenan injection volume was subtracted from the collected fluid volume to give the volume of exudate fluid. The number of cells infiltrating into the pouch fluid was determined by carrying out total cell counts which were performed with a Haemocytometer. Smears of exudate cells were stained with Haematoxylin and Eosin (HE) (Appendix IV), and differentially counted for PMNL and MNL. Specimens of pouch tissue were excised for histological examination. Tissues were fixed in 10% formal saline, embedded in resin, cut at 5 µm sections (Appendix III) and stained with HE. Stained slides were viewed under an Olympus light microscope.

5.4.1.2 STUDY OF CHRONIC INFLAMMATION IN THE RAT SUBCUTANEOUS AIR-POUCH MODEL

Chronic inflammation was induced in 5 sets of 3 Wistar rats with 6-day old subcutaneous air-pouches (formed as outlined in Section 5.3.2.1), by the injection of 2 ml 2% w/v sterile carrageenan solution. Groups of animals were sacrificed 1, 3, 7, 10,

and 14 days after carrageenan challenge. The same characterization procedures were carried out as outlined in Section 5.4.1.1.

5.4.2 DETERMINATION OF INCREASED VASCULAR PERMEABILITY AND BLOOD VESSEL FORMATION IN SUBCUTANEOUS AIR-POUCH TISSUES WITH ACUTE AND CHRONIC INFLAMMATION

Elevation of the permeability to macromolecules in local vascular networks is recognized as one of the most prominent features in the inflammatory process (Persson & Svensjö, 1985). Clinical symptoms such as tissue oedema and fluid accumulation in body cavities results principally from the elevation of vascular permeability in the local tissues. Therefore, it is feasible that microspheres might also be able to permeate the vasculature at such sites, depending on the degree of inflammation and damage to the local vasculature. The following two studies attempted to show the increase in vascular permeability and any increase in blood vessel formation in the acute and chronic stages of carrageenan-induced inflammation in the subcutaneous air-pouch model.

5.4.2.1 STUDY OF INCREASED VASCULAR PERMEABILITY USING RADIOIODONATED SERUM ALBUMIN AS A MARKER OF VASCULAR PERMEABILITY

Using [125 I]-BSA as a marker, and based on the method of Tsurufuji *et al.*, (1978), this study allowed a determination of the increase in vascular permeability in the following tissues; 1) in control air-blebs, 2) in air-pouches without irritant, 3) in air-pouches induced with acute inflammation and, 4) in air-pouches induced with chronic inflammation.

12 male Wistar rats (150 g) were divided into 4 groups of 3 rats. Air-blebs were formed in Group 1 rats. 6-day old subcutaneous air-pouches were formed as outlined in Section 5.3.2 in Group 2 rats. Acute inflammation was induced in 6-day old subcutaneous air-pouches as described in Section 5.3.2.1 in Group 3 rats, and chronic inflammation was induced in 6-day old subcutaneous air-pouches as described in Section 5.3.2.2 in Group 4 rats.

[125 I]-BSA was used at a concentration of 0.185 MBq ml⁻¹ in physiological saline. The dose injected was approximately 0.037 MBq in 200 μ l. The initial radioactivity of the injection dose was measured immediately prior to tail vein injection.

Animals were anaesthetized by i.m. injection of Hypnorm. The tail vein was warmed by the heat of a table lamp for 5-10 min to cause mild vasodilation, thereby facilitating visualization of the tail vein. A 25-gauge needle was bent slightly and introduced just below the surface of the skin along the line of the tail vein. Once in the vein, blood was gently massaged back into the needle, the syringe attached and the radiolabelled dose slowly administered.

After injection, the radiolabelled serum albumin was allowed to equilibrate in the circulation for 30 min before the animals were sacrificed by CO₂ asphyxiation. Control and pouch tissues were excised, weighed and analysed for radioactivity. As a further control, skin samples were stripped from the abdomen of each animal, weighed and analysed for their radioactivity content.

In order to calculate the exact radioactivity of the administered dose, the radioactivity of the used Eppendorf tube, needle and syringe was counted, and the administered dose calculated according to the following formula:

$$\text{Radioactivity of administered dose} = \text{Initial radioactivity of dose} - \text{Radioactivity of Eppendorf, needle and syringe.}$$

5.4.2.2 STUDY OF INCREASED BLOOD VESSEL FORMATION IN AIR-POUCH TISSUE BY DIRECT EXAMINATION

In order to view any increased blood vessel formation in the air-pouch challenged with 2ml 2% w/v carrageenan, air-bleb and control, acute and chronic air-pouch tissues were excised and pinned inside face upwards on a cork board. The tissues were photographed immediately after excision, since leakage of blood from the vessel walls occurs rapidly, obscuring the clarity of blood vessel visualization. Air-bleb tissue and air-pouch tissue without irritant injection were used as controls for comparison of blood vessel formation in the inflamed tissues.

5.4.3 DETERMINATION OF ALBUMIN MICROSPHERE DISTRIBUTION TO SUBCUTANEOUS AIR-POUCH TISSUES WITH ACUTE AND CHRONIC INFLAMMATION FOLLOWING ORAL AND INTRAVENOUS ADMINISTRATION

The tissue distribution of albumin microspheres to various organs of the body and to the site of inflammation, after oral and intravenous administration, was quantified using [¹²⁵I]-labelled albumin microspheres with a geometric mean diameter and σ g of

2.67±1.28 µm. The distribution pattern of uncoated albumin microspheres, as well as Poloxamine 908-coated albumin microspheres, was investigated in experimental animals with 1) control air-blebs, 2) air-pouches with no irritant, 3) air-pouches with acute inflammation and 4) air-pouches with chronic inflammation. Each set was composed of 3 experimental animals.

5.4.3.1 INTRAVENOUS ADMINISTRATION

[¹²⁵I]-labelled albumin microspheres were prepared as outlined in Section 5.3.3, weighed in Eppendorf tubes and their radioactivity measured prior to use. Each dose contained approximately 15 mg microspheres and had a radioactivity of about 0.05 MBq. Immediately prior to administration, the microspheres were suspended in 250 µl PBS, vortexed thoroughly and pulled up into a 1 ml syringe. The experimental animals were lightly anaesthetized with halothane and the microspheres administered by tail vein injection. The radioactivity of the administered dose was calculated as described in Section 5.4.2.

4 hr after dosing the animals were sacrificed by CO₂ asphyxiation. Blood samples were collected by cardiac puncture, and their volume and radioactivity measured. Organs of interest, namely the liver, lungs, spleen, air-pouch tissue and fluid, and abdomen skin were excised, weighed and their radioactivity measured.

As a control, the amount of free radioactivity not bound to the microspheres was measured. A sample of microspheres of known radioactivity was incubated at 37°C in a Tecam 5B-16 shaking water bath (Techne Ltd., Cambridge, UK) for 4 hr. The microspheres were removed by centrifugation and the radioactivity of aliquots of the supernatant measured. The free radioactivity was then expressed as a percentage of the initial microsphere radioactivity.

5.4.3.2 ORAL ADMINISTRATION

[¹²⁵I]-labelled albumin microspheres were prepared as outlined in Section 5.3.3, weighed in Eppendorf tubes and their radioactivity measured prior to use. Each dose contained approximately 15 mg microspheres and had a radioactivity of about 0.05 MBq. Immediately prior to administration, the microspheres were suspended in 1 ml physiological saline, vortexed thoroughly and pulled up into a 1 ml syringe.

The experimental animals were starved overnight and lightly anaesthetized with halothane before the microspheres were administered by gavage needle. The radioactivity of the administered dose was calculated as described in Section 5.4.2. Animals were kept in metabolism cages, with water *ad libitum* but no food, to collect urine and faecal samples.

Sets of experimental animals were sacrificed by CO₂ asphyxiation, 4 hr and 24 hr after dosing. Blood samples were collected by cardiac puncture, and their volume and radioactivity measured. Organs of interest, namely the stomach, intestine, colon, faeces, urine, kidney, liver, lungs, spleen, air-pouch tissue and fluid, and abdomen skin were excised, weighed and their radioactivity measured.

As a control, the amount of free radioactivity not bound to the microspheres was measured. A sample of microspheres of known radioactivity was incubated at 37°C in a Tecam 5B-16 shaking water bath (Techne Ltd.) for 4 hr and 24 hr. The microspheres were removed by centrifugation and the radioactivity of aliquots of the supernatant measured. The free radioactivity was then expressed as a percentage of the initial microsphere radioactivity.

5.5 RESULTS AND DISCUSSION

5.5.1 CHARACTERIZATION OF ACUTE AND CHRONIC INFLAMMATION INDUCED IN SUBCUTANEOUS AIR-POUCH TISSUES

Subcutaneous air-pouches were routinely used 6 days after the initial injection of air. It was felt that it was an advantage to maintain a constant protocol for the formation and use of the air-pouch in order to minimize the possibility of time-related variables. Sedgwick *et al.*, (1983), and Sin *et al.*, (1984c), both showed that the reactivity of the air-pouch to a variety of stimuli, assessed by the volume of exudate and number of cells accumulating, changed dramatically in air-pouches of different ages. Thus, 1-day old pouches produced little or no response, while 3-day old pouches responded well, and 6-day old pouches produced even larger increases in cell accumulation and the volume of inflammatory exudate. In contrast to these results, Tsurufuji and co-workers (Konno & Tsurufuji, 1983; Tsurufuji *et al.*, 1982; Tsurufuji *et al.*, 1978), routinely performed experiments only 24 hr after a single s.c. injection of air to form a pouch, and observed increases in cell accumulation and volume of inflammatory exudate. The difference between these observations may be related to the different inflammatory stimuli used by the two groups. In addition, Sedgwick and associates injected their

stimuli in physiological saline, while Tsurufuji and co-workers used carboxymethyl cellulose as a carrier to prevent too-rapid absorption of the injected stimuli. Clearly, the conditions under which the pouch are used will have a large influence on the response of the pouch. Thus, extreme care was taken to standardize conditions, not only in formation of the subcutaneous air-pouches and their use 6 days after the initial injection of air, but also in the development of irritant induced inflammation.

The inflammatory response of 6-day old air-pouches to the injection of 2 ml 2% sterile carrageenan in physiological saline solution was monitored in terms of exudate volume and the number of infiltrating cells. Figure 5.1 shows the volume of pouch fluid collected from 2 hr to 10 days after carrageenan injection. It was initially anticipated to continue the study for 14 days post carrageenan injection, but the animals were sacrificed on day 10 due to the excessively large volumes of fluid being produced. Over the first 24 hr, the fluid volume increased slowly to around 3 ml at 24 hr. This volume corresponds to that reported by Sedgwick *et al.*, (1983), who injected a 1% w/v carrageenan in physiological saline solution. After 24 hr, fluid volume increased rapidly, reaching > 60 ml on day 10.

FIGURE 5.1 *Pouch Fluid Exudate Volumes (▲) Collected Over A 10 Day Period From Rat Subcutaneous Air-Pouches Injected With 2 ml 2% w/v Sterile Carrageenan Solution. Each value represents the mean \pm sd (n=3 rats)*

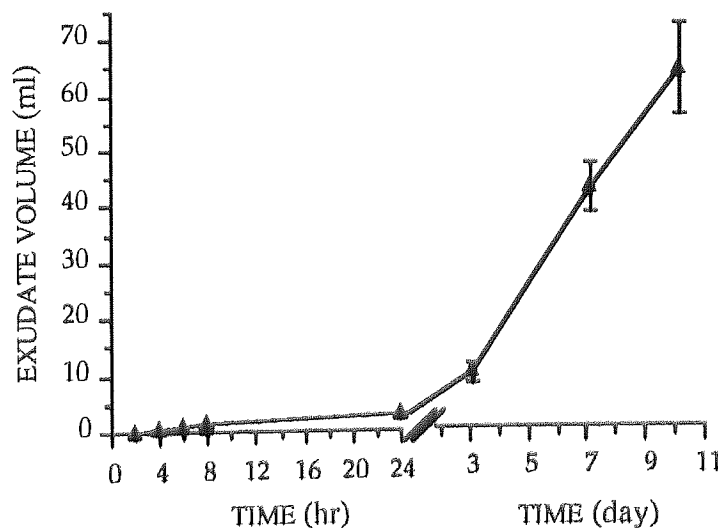


Figure 5.2 shows the total number of cells infiltrating into the 6 day old air-pouch up to 24 hr after the injection of 2 ml 2% carrageenan in physiological saline solution. Initially, >98% of these infiltrating cells were PMNL, as shown by slide smears of

FIGURE 5.2 *Total Cell Numbers (\blacktriangle) And % PMNL (\triangle) In Pouch Fluid Exudates Collected From Rat Subcutaneous Air-Pouches 2, 4, 6, 8, And 24 Hours After The Injection of 2 ml 2% w/v Sterile Carrageenan Solution. Each value represents the mean \pm sd (n=3 rats)*

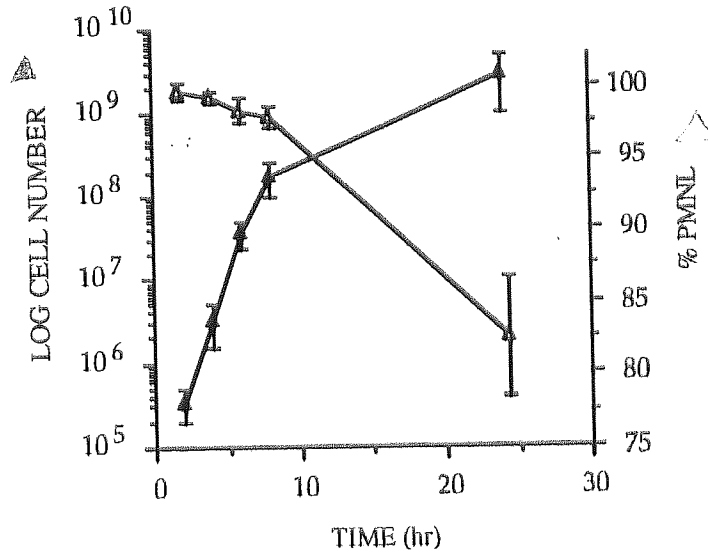
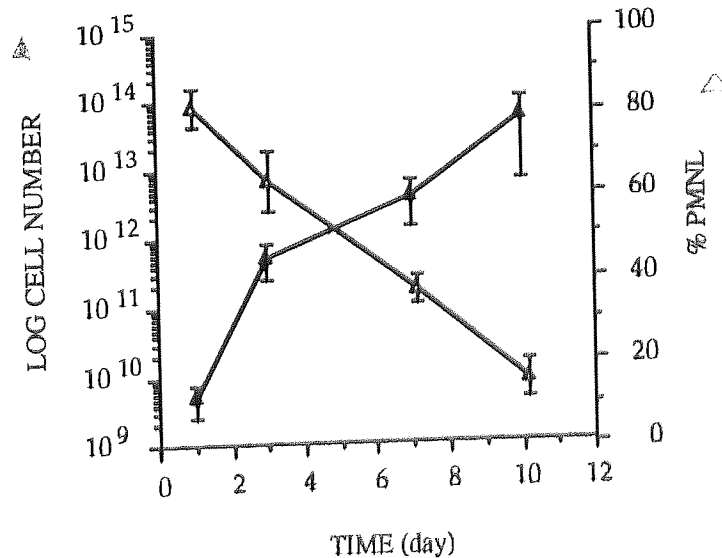


FIGURE 5.3 *Total Cell Numbers (\blacktriangle) And % PMNL (\triangle) In Pouch Fluid Exudates Collected From Rat Subcutaneous Air-Pouches 1, 3, 7, And 10 Days After The Injection of 2 ml 2% w/v Sterile Carrageenan Solution. Each value represents the mean \pm sd (n=3 rats)*



fluid exudate stained with HE, and by 24 hr, the proportion of PMNL had dropped to 83%, with the increased appearance of MNL. Histological examination of 5 μ m sections of pouch tissue, stained with HE, 24 hr post-carrageenan injection, revealed a structure containing four to ten layers of fibroblast-like cells overlying the vascular connective tissue. Predominately PMNL are seen infiltrating the fibroblast-like layers along with vacuolated macrophages. This is in comparison with 6-day old air-pouch tissue which had a lining layer 2-3 cells thick, composed of fibroblast-like cells. The infiltration of PMNL in response to an inflammatory stimuli is indicative of acute inflammation, and 6-day old pouches challenged with carrageenan were routinely used 24 hr post injection.

Figure 5.3 shows a continuing increase in the number of infiltrating cells 10 days after carrageenan injection, with a continuing decrease in the percentage of PMNL to around 17%. Histological examination of 5 μ m sections, stained with HE, of tissues 3 days post-carrageenan injection, revealed an increase to about 20 layers of fibroblast-like cells infiltrated with PMNL and vacuolated macrophages. This thick granulomatous tissue formation is indicative of chronic inflammation, and air-pouches were routinely used 3 days after carrageenan injection for chronic inflammation studies.

5.5.2 DETERMINATION OF INCREASED VASCULAR PERMEABILITY AND BLOOD VESSEL FORMATION IN SUBCUTANEOUS AIR-POUCH TISSUES

Blood vessel formation in control air-bleb, air-pouch, acutely inflamed air-pouch and chronically inflamed air-pouch tissues are shown in Plates 5.1, 5.2, 5.3 and 5.4 respectively. Gross examination of the exposed 6-day old air-pouch tissue revealed the presence of large blood vessels running through the pouch tissue. As the pouch tissues developed acute, followed by chronic inflammation after irritant administration, there is seen to be an increase in blood vessel prominence and formation. Vascular permeability was also increased as can be seen by the leakage of blood from the vessels in the inflamed tissues compared to the control air-bleb and air-pouch tissues.

Since the vessels of the microcirculation in the air-pouch tissues are newly formed, it was thought that these tissues may either leak non-specifically or fail to leak in response to inflammatory stimuli. To answer these questions, a study of the vascular permeability within each of the control and inflamed air-pouch tissues was undertaken using [125 I]-BSA as a blood study marker. The results are shown in Table 5.1. The results suggest that there is a significant increase in vascular permeability in both the inflamed acute and chronic air-pouch tissues, when compared to the control air-bleb

tissue, but no significant increase in vascular permeability is seen in the air-pouch tissue with no irritant administered. Thus, the newly formed vasculature of the air-pouch does not appear to be leaking non-specifically, and the increased permeability in the inflamed tissues indicates that the blood vessels are responding in a typical fashion to the inflammatory mediator, carrageenan (Di Rosa, 1972). These results also correlate with the gross examination of blood vessel formation in the air-pouch tissues as discussed earlier. Interestingly, there appears to be no significant difference in the venular permeability of the inflamed air-pouch tissues when compared to the abdomen tissues taken from the same animals. This seems to suggest a systemic effect of the administered carrageenan, as well as an enhanced localized effect within the air-pouch.

TABLE 5,1 *The Percentage Of Dosed Radioactivity Per Gram Of Tissue In: (1) Control Air-Bleb Tissue, (2) Air-Pouch Tissue With No Irritant Administered, (3) Air-Pouch Tissue 24 Hours After The Injection Of 2 ml 2% w/v Sterile Carrageenan (Acute Inflammation), And (4) Air-Pouch Tissue 72 Hours After The Injection Of 2 ml 2% w/v Sterile Carrageenan (Chronic Inflammation). Each value represents the mean \pm sd (n=3 rats). ¹Significantly different from air-bleb tissue. * $P \leq 0.005$*

TYPE OF AIR-POUCH FORMED	CONTROL SKIN TISSUE % OF DOSED RADIOACTIVITY g^{-1}	POUCH TISSUE % OF DOSED RADIOACTIVITY g^{-1}
AIR-BLEB	0.146 \pm 0.016	0.157 \pm 0.024
AIR-POUCH NO IRRITANT	0.298 \pm 0.067	0.353 \pm 0.103
AIR-POUCH- ACUTE INFLAMMATION	0.266 \pm 0.013	*10.382 \pm 0.027
AIR-POUCH- CHRONIC INFLAMMATION	0.493 \pm 0.087	*10.675 \pm 0.054

PLATE 5.1 *Blood Vessel Formation In Subcutaneous Air-Bleb Tissue*

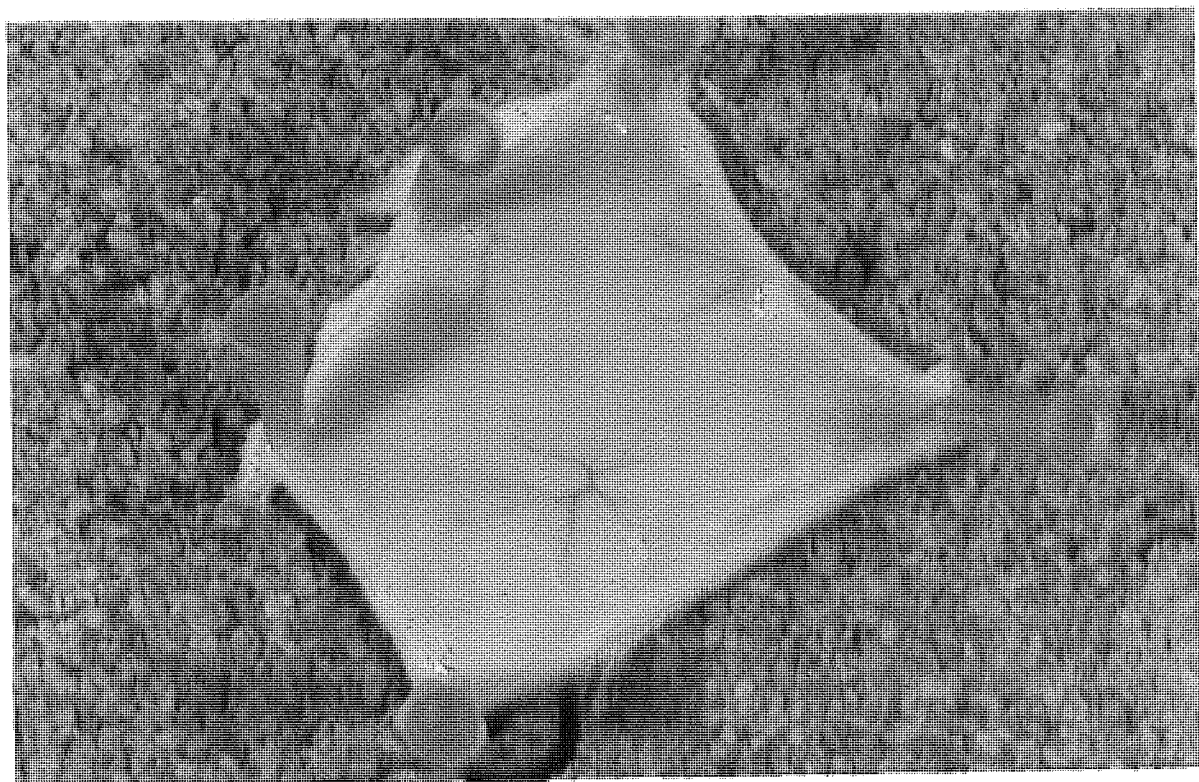


PLATE 5.2 *Blood Vessel Formation In Subcutaneous Air-Pouch Tissue*



PLATE 5.3 *Blood Vessel Formation In Subcutaneous Air-Pouch Tissue 24 Hours After Injection Of 2 ml 2% w/v Sterile Carrageenan Solution*

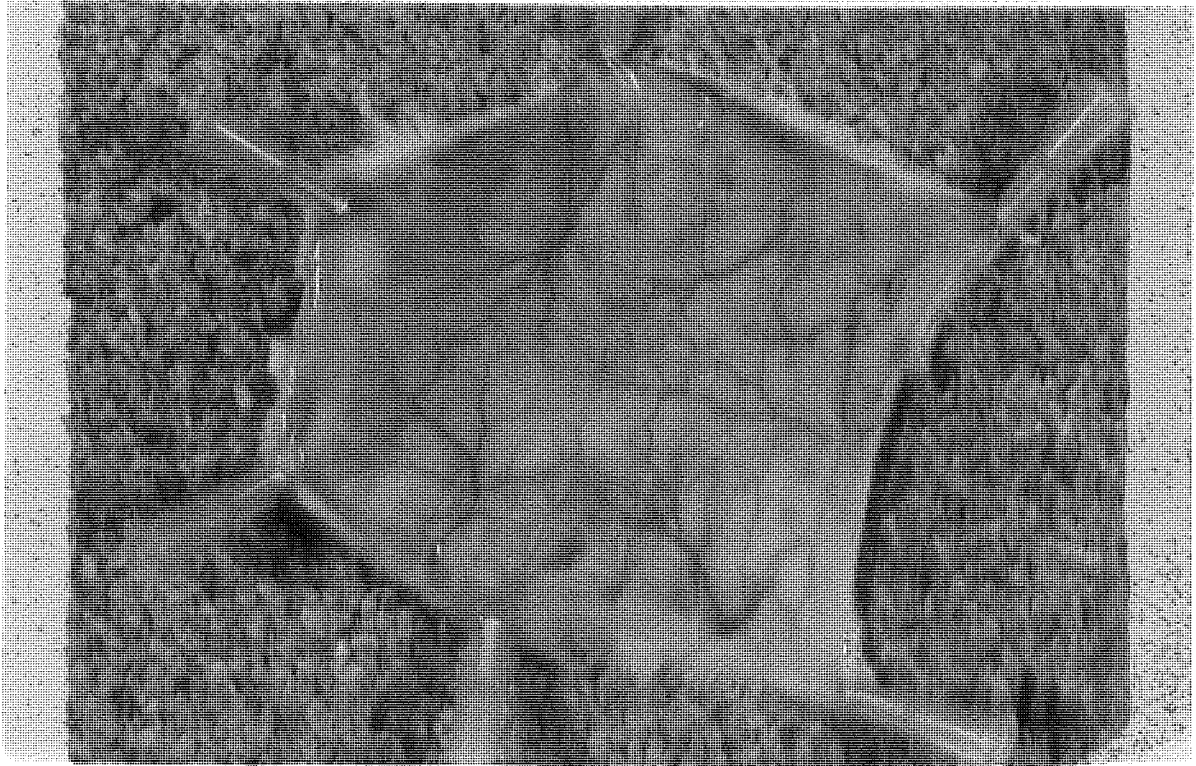
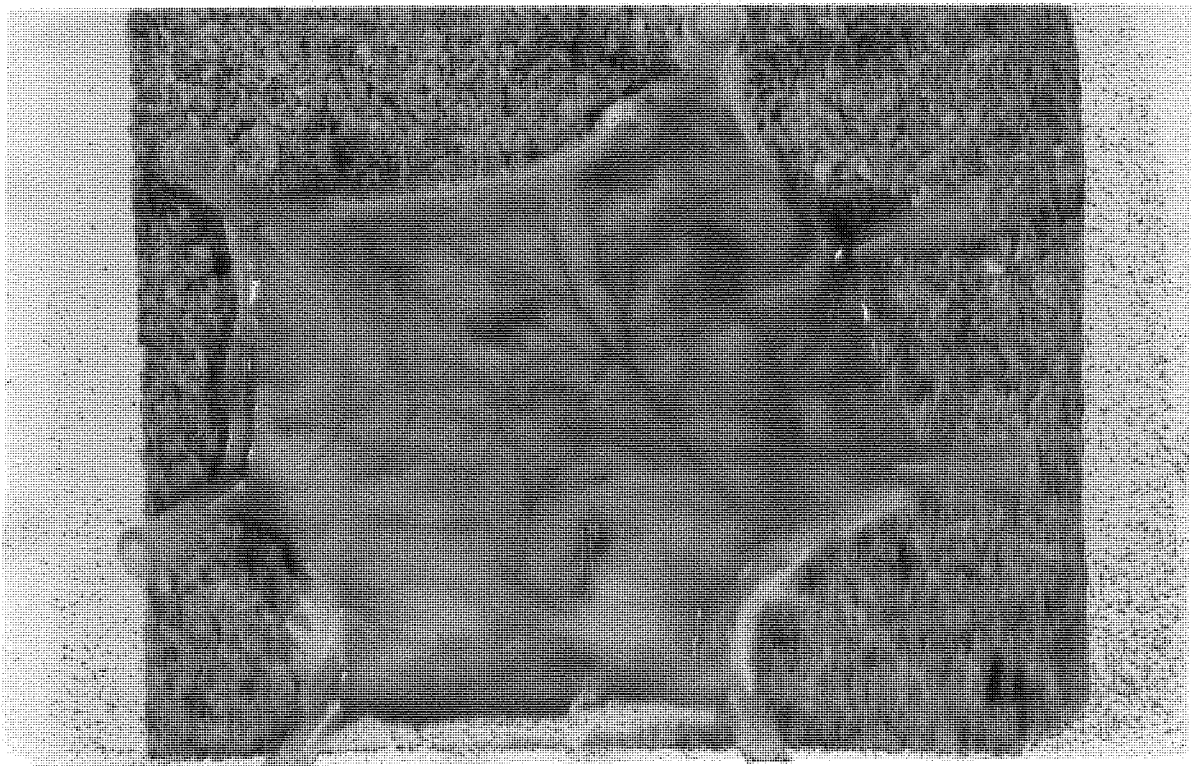


PLATE 5.4 *Blood Vessel Formation In Air-Pouch Tissue 72 Hours After Injection Of 2 ml 2% w/v Sterile Carrageenan Solution*



5.5.3 TISSUE DISTRIBUTION OF ORALLY AND INTRAVENOUSLY ADMINISTERED ALBUMIN MICROSPHERES

Albumin microspheres were successfully radiolabelled using commercially available [¹²⁵I]-BSA in the microsphere manufacturing process. After washing, the free [¹²⁵I] was negligible at <1% after 4 hr incubation at 37°C, and <1.5% after 24 hr incubation at 37°C. However, this does not account for the possible degradation of the radiolabelled albumin microspheres by proteases, found in the blood and GIT, which may occur during *in vivo* microsphere distribution studies. The labelled microspheres were coated with Poloxamine 908, with zeta potential values being reduced from -19.4 mV to -1.24 mV (Section 2.5.3).

The administered radioactive doses for all tissue distribution experiments were calculated by subtracting the remaining dose in the needle, syringe and Eppendorf tube after administration, from the initial radioactive dose in the Eppendorf tube immediately prior to administration. Thus, it was attempted to prevent unusually low microsphere recovery percentages, and obtain more accurate distribution values, since it was impossible to administer all of the initial dose without some loss due to vessel transfer.

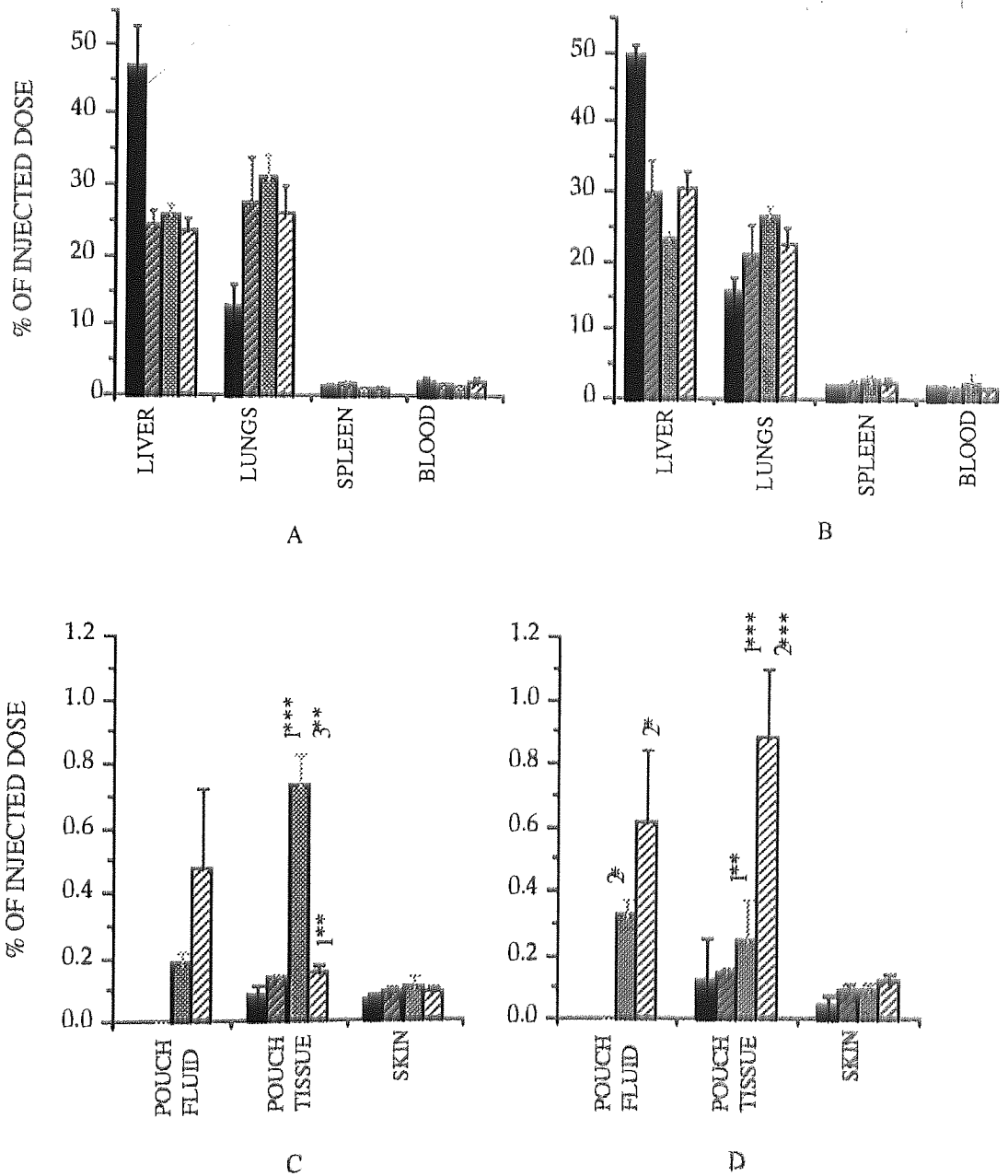
5.5.3.1 TISSUE DISTRIBUTION OF INTRAVENOUSLY ADMINISTERED ALBUMIN MICROSPHERES

The tissue distributions of the percentage of administered radioactive doses of non-coated and Poloxamine 908-coated radiolabelled albumin microspheres, 4 hours after i.v. administration to rats with 1) control air-blebs, 2) air-pouches without irritant injection, 3) acutely inflamed air-pouches and 4) chronically inflamed air-pouches, are shown in Figure 5.4. All results are expressed as the percentage of injected radioactive dose recovered from the whole tissues, except for the pouch tissue and skin tissue, both of which are expressed as the percentage of dose per gram of tissue. The collection and weighing of the whole pouch tissues showed variation in the mean weight, and it was proposed that a percentage dose per gram would be a more accurate method of assessing the distribution of microspheres to pouch tissues.

The tissue distributions for non-coated albumin microspheres, 4 hr after i.v. administration to rats with control and inflamed air-pouches as described above, are shown in Figure 5.4, A. Administration of radiolabelled microspheres to rats with control air-blebs, shows 46.8% of the administered radioactive dose in the liver, 12.78% in the lungs, 1.2% in the spleen, and 1.76% circulating in the blood. This

FIGURE 5.4 Tissue Distribution Of [¹²⁵I]-Albumin Microspheres (geometric mean diameter and σ $2.67 \pm 1.28 \mu\text{m}$) 4 Hours After Intravenous Administration Via Tail Vein Injection. A And C - Microspheres With No Coating. B And D - Microspheres Coated With Poloxamine 908. Each bar represents the mean \pm sd (n=3 rats).¹Significantly different from control tissue. ²Significantly different from plain microspheres. ³Significantly different from Poloxamine 908-coated microspheres. *P<0.05 **P<0.01 ***P<0.005

ANIMALS WITH; ■ AIR-BLEB ▨ AIR-POUCH ▩ ACUTE AIR-POUCH ▪ CHRONIC AIR-POUCH



distribution indicates the microspheres are being removed from the circulation by the Kupffer cells in the liver, with a smaller percentage being removed by the filtering capacity of the lungs. This tissue distribution is different from that seen in the rats induced with air-pouches, acutely inflamed air-pouches and chronically inflamed air-pouches, where a greater percentage of the administered radioactive dose is seen in the lungs, around 27%, with a smaller percentage, around 25%, in the liver. This indicates aggregation of the microspheres prior to injection, giving rise to in effect larger particles, which are removed by the filtering capacity of the lungs. The percentage of injected radioactive doses recovered from the spleen and blood for all rats was very similar, around 1% in the spleen, and around 1.5% circulating in the blood.

The percentage of the injected radioactive doses recovered in pouch fluids and tissues are shown in Figure 5.4, C. No pouch fluid was collected from rats with control air-blebs and air-pouches without irritant injection. The percentage of injected radioactive doses recovered in air-bleb and air-pouch tissues were 0.08% and 0.13% respectively, and were not significantly different from those recovered in control skin tissues, analysed statistically by paired Student's t-tests. However, statistically significant higher percentages of the injected radioactive doses (analysed by paired Student's t-tests) were recovered from acutely inflamed ($P < 0.005$) and chronically inflamed ($P < 0.01$) air-pouch tissues, with 0.73% and 0.47% of injected radioactive doses respectively being recovered per gram. Thus, it would appear that albumin microspheres were able to leave the vascular compartment at the site of inflammation.

A similar tissue distribution pattern to that of non-coated albumin microspheres was seen for Poloxamine 908-coated albumin microspheres in the liver, lung, spleen and blood tissues, shown in Figure 5.4, C. No significant difference was seen in the ability of Poloxamine 908-coated albumin microspheres to remain in the circulation longer than non-coated microspheres after 4 hr. Again, significantly higher percentages of the injected radioactive doses were recovered from acutely inflamed ($P < 0.01$) and chronically inflamed ($P < 0.005$) air-pouch tissues compared to control skin tissues (analysed by paired Student's t-test) (Figure 5.4, D). Using unpaired Student's t-tests it was shown that the percentage radioactive doses recovered from the chronically inflamed pouch tissues ($P < 0.005$), and acutely inflamed ($P < 0.01$) and chronically inflamed ($P < 0.01$) air-pouch fluids, were significantly higher than those obtained for non-coated albumin microspheres.

Thus, in this study, the ability of albumin microspheres, (geometric mean diameter $2.67 \pm 1.28 \mu\text{m}$) coated with Poloxamine 908, to reach sites of inflammation was

compared to that of non-coated albumin microspheres. It has been shown that microspheres coated with block-copolymers remain in the circulation for comparatively longer periods of time (Wright & Illum, 1992), and therefore have a greater chance of migrating from the vasculature and accumulating at inflamed tissue sites (Illum *et al.*, 1989). The results for non-coated albumin microspheres show <0.5% of the injected radioactive doses of albumin microspheres were recovered from the pouch fluids of acutely and chronically inflamed pouch tissues, with 0.73% and 0.17% being recovered respectively per gram of the pouch tissue (Figure 5.4, C). In comparison, radioactive doses of Poloxamine 908-coated albumin microspheres were recovered from the pouch fluids of acutely and chronically inflamed pouch in significantly, if only slightly greater amounts, with 0.27% and 0.9% being recovered respectively per gram of pouch tissue (Figure 5.4, D). The weight of air-pouch tissue varied greatly between animals so a total percentage of the injected radioactive dose was not made. However, if an average pouch was taken as 5 g, then multiplying the percentage of injected Poloxamine 908-coated albumin microsphere radioactive dose recovered from chronically inflamed tissues, about 4.5% of the injected radioactive doses were found in chronically inflamed air-pouch tissues. Similarly, about 1.4% of the injected radioactive doses were found in acutely inflamed tissues. The results obtained in the above studies may be contrasted with those obtained by Alpar *et al.*, (1989). This group has previously reported the accumulation of 1.1 μm latex microspheres in acutely inflamed air-pouch tissues 4 hr after direct injection into the heart. Between 0.26% - 4.76% of the latex particle doses administered were reported to be found in acutely inflamed air-pouches. The wide variation in these results was explained by the rapid removal of particles from the blood within 10 min after dosing. The greater the removal of particles from the blood, the less microsphere accumulation was found in the air-pouch tissues.

Inflammation causes major changes at the level of terminal circulation, including severe local damage to the vascular endothelium and the basement membrane (Persson & Svenjö, 1985). This situation is found in all sites where leukocyte diapedesis into the granulation tissue takes place, and where, as a consequence, the mechanical stress of leukocyte passage induces discontinuity of the basement membrane causing defects as large as 1-4 μm (Schoefl, 1964). If both the endothelium and the basement membrane become disrupted, intravenously injected particulates have been shown to extravasate into the extracellular space (Alpar *et al.*, 1989; De Schijver *et al.*, 1987; Illum *et al.*, 1987; Love *et al.*, 1989). Using the subcutaneous air-pouch model of inflammation, it has been shown that leukocyte infiltration into the pouch cavity occurs upon injection of an inflammatory stimulus, carrageenan, along with increased vascular permeability of the local capillary network. Intravenous administration of albumin microspheres

(geometric mean diameter and σ_g $2.67 \pm 1.28 \mu\text{m}$) has shown the accumulation of such microspheres at the site of inflammation, indicating their passage through disrupted endothelium and basement membranes into the inflamed tissues.

5.5.3.2 TISSUE DISTRIBUTION OF ORALLY ADMINISTERED ALBUMIN MICROSPHERES

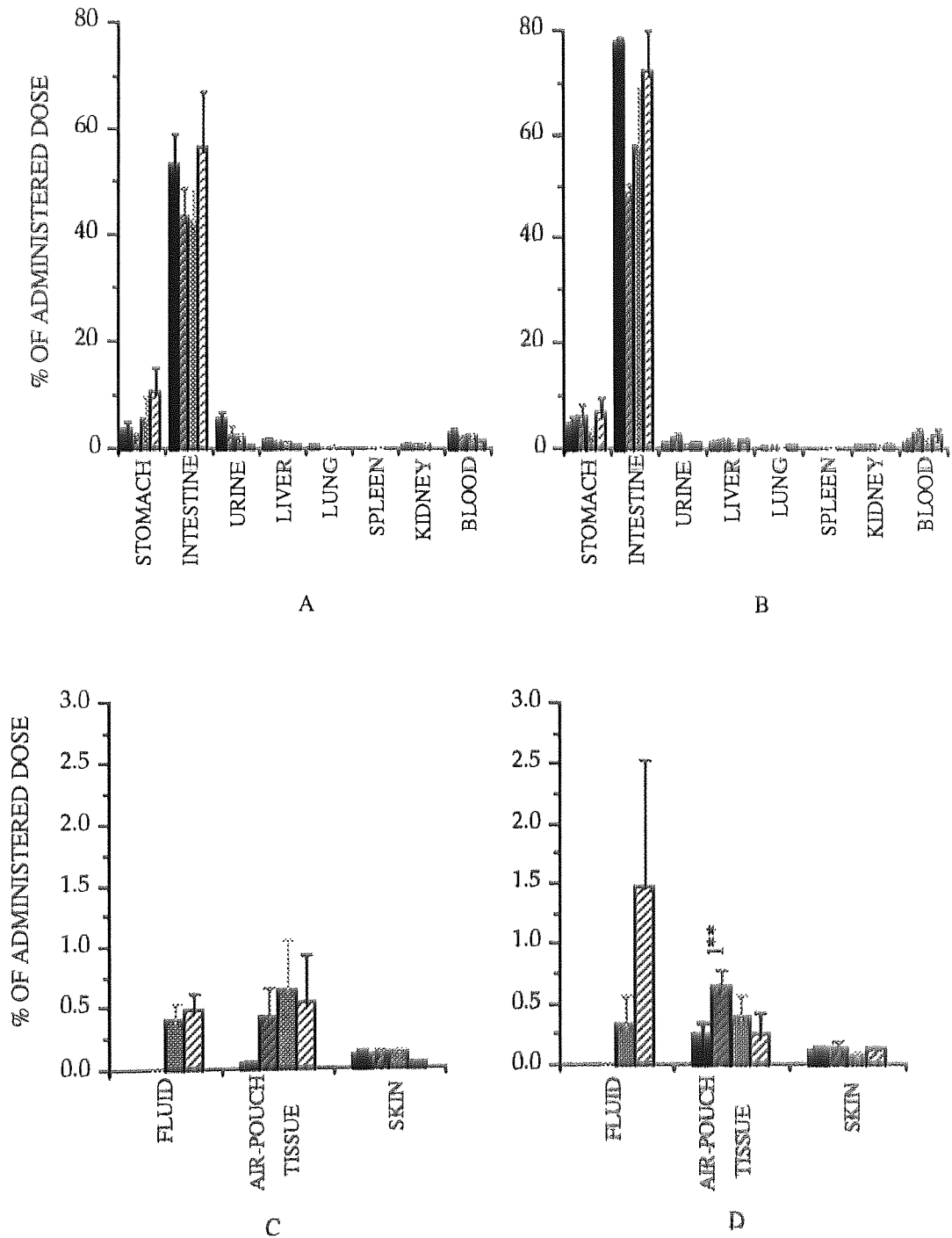
The tissue distributions of radiolabelled albumin microspheres and Poloxamine 908-coated radiolabelled albumin microspheres, 4 and 24 hours after oral administration by gavage to rats with 1) control air-blebs, 2) air-pouches without irritant injection, 3) acutely inflamed air-pouches and 4) chronically inflamed air-pouches, are shown in Figures 5.5 and 5.6 respectively. All results are expressed as the percentage of the radioactive dose recovered from the whole tissue, except for the pouch tissue and skin tissue, both of which are expressed as the percentage of dose per gram tissue.

The tissue distributions of non-coated and Poloxamine 908-coated [^{125}I]-labelled albumin microspheres, 4 hr after oral administration, are shown in Figure 5.5. In the case of non-coated albumin microspheres, about 50% of the radioactive dose is found in the intestine, with about 5% remaining in the stomach. In contrast to this, Poloxamine 908-coated albumin microspheres show similar levels of radioactive dose in the stomach but higher levels in the intestine. This may be due to a difference in the rate of intestine mobility upon the day of experimentation. About 0.3% of the radioactive dose was found in the kidney in both non-coated and Poloxamine 908-coated microsphere studies, with about 2% being found in the urine.

Oral uptake of both non-coated and Poloxamine 908-coated particles is suggested by the presence of radioactivity in the blood, liver and skin tissues of all the experimental rats, since these tissues are physically separate from those of the GIT, and microspheres must have penetrated the epithelial cell barrier to reach them. Negligible amounts of radioactivity were found in the lung and spleen in all experimental studies. Total oral uptake for both non-coated and Poloxamine 908-coated microspheres was about 10-15% of the radioactive dose, with similar levels in control air-bleb rats and those induced with pouches of different inflammatory states. About 0.5% of the radioactive dose was detected in air-pouch tissues, with only Poloxamine 908-coated particles being significantly higher ($P < 0.01$) in non-inflamed air-pouch tissue than in control skin tissues, as analysed by paired Student's t-test.

FIGURE 5.5 Tissue Distribution Of [125 I]-Albumin Microspheres (geometric mean diameter and σ $2.67 \pm 1.28 \mu\text{m}$) 4 Hours After Oral Administration By Gavage Needle. A And C - Microspheres With No Coating. B And D - Microspheres Coated With Poloxamine 908. Each bar represents the mean \pm sd ($n=3$ rats). ¹Significantly different from control tissue. ** $P < 0.01$

ANIMALS WITH; ■ AIR-BLEB ▒ AIR-POUCH ▒ ACUTE AIR-POUCH ▒ CHRONIC AIR-POUCH



The tissue distributions of non-coated and Poloxamine 908-coated [^{125}I]-labelled albumin microspheres, 24 hr after oral administration, are shown in Figure 5.6. After 24 hrs, between 40-60% of the recovered radioactive dose is excreted in the faeces for both non-coated and Poloxamine 908-coated microspheres, with about 2% being retained within the stomach and about 5% in the intestine. After 24 hrs, compared to 4 hr post oral dosing, a larger percentage of the radioactive dose, between 5-10%, was found in the urine of both non-coated and Poloxamine 908-coated microsphere studies, with a similar amount, 0.2%, being recovered from the kidneys. The presence of albumin microspheres in the urine was suggested by visual examination of urine specimens collected from all the experimental rats, and samples were taken for further examination by SEM. Urine samples were centrifuged (13,000 rpm, 5 min), the pellets were washed in water, freeze-dried and examined by SEM. Examination of the pellets collected from urine samples from both non-coated and Poloxamine 908-coated albumin microsphere dosed rats, showed the presence of spherical, non-biodegraded albumin microspheres amongst urate crystals, an example of which is shown in Plate 5.5.

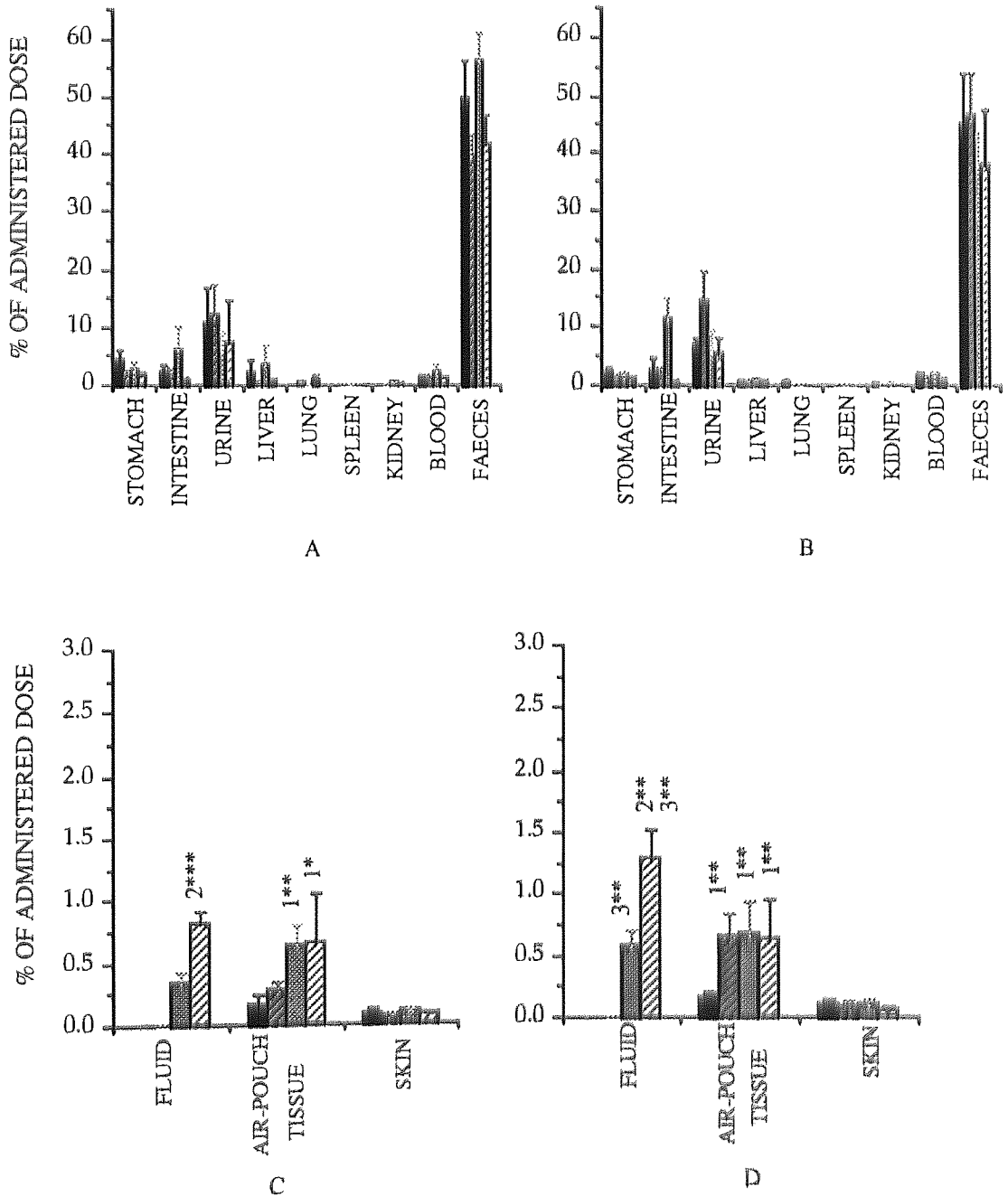
Oral uptake of both non-coated and Poloxamine 908-coated particles is suggested by the presence of radiolabel in the blood, liver, and skin tissues of all the experimental rats, since these tissues are physically separate from those of the GIT, and microspheres must have penetrated the epithelial cell barrier to reach them. Negligible amounts of radioactivity were found in the lung and spleen in all experimental studies 24 hr post administration. Again, total oral uptake for both non-coated and Poloxamine 908-coated microspheres was about 10-15% of the administered dose, with similar levels in control air-bleb rats and those induced with pouches of different inflammatory states. About 0.6% of the radioactive dose of non-coated albumin microspheres was detected in acutely and chronically inflamed air-pouch tissues, being significantly different from control skin tissues, analysed statistically by paired Student's t-tests. Similarly, about 0.6% of the administered dose of Poloxamine 908-coated albumin microspheres was detected in the acutely and chronically inflamed air-pouch tissues as well as in the air-pouch tissue with no irritant injected, and again, were found to be significantly higher from the percentage of radioactive dose in the control skin tissues. The percentage of the radioactive dose of non-coated albumin microspheres in the chronically inflamed air-pouch was significantly higher than in the acutely inflamed air-pouch and this was also the case in the Poloxamine 908-coated microspheres, as analysed by paired Student's t-tests. The percentage of administered dose of Poloxamine 908-coated albumin microspheres was significantly higher in both acute and chronic air-pouch fluids than the levels detected in non-coated albumin microsphere

FIGURE 5.6 Tissue Distribution Of [¹²⁵I]-Albumin Microspheres (geometric mean diameter and σ $2.67 \pm 1.28 \mu\text{m}$) 24 Hours After Oral Administration By Gavage Needle. A And C - Microspheres With No Coating. B And D - Microspheres Coated With Poloxamine 908.

Each bar represents the mean \pm sd (n=3 rats).¹Significantly different from control tissue. ²Significantly different from acute fluid.

³Significantly different from plain microspheres. *P<0.1 **P<0.05 ***P<0.005

ANIMALS WITH; ■ AIR-BLEB ■ AIR-POUCH ■ ACUTE AIR-POUCH ▨ CHRONIC AIR-POUCH



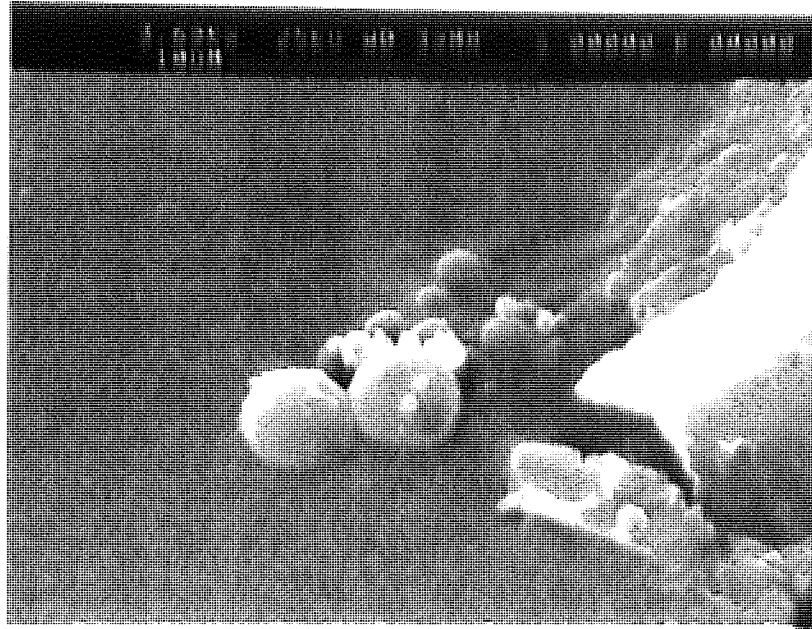
fluids than the levels detected in non-coated albumin microsphere studies, analysed by unpaired t-tests.

The uptake of albumin microspheres (geometric mean diameter $2.67 \pm 1.28 \mu\text{m}$) across the GIT was suggested, with results showing about 10-15% uptake of the radioactive dose, 4 hr post administration and about 4% 24 hr post administration. No difference in the uptake was seen between Poloxamer 908-coated and non-coated albumin microspheres. Alpar *et al.*, (1989) and Lewis *et al.*, (1992), also reported the co-administration of Poloxamer-908 with orally dosed latex microspheres ($1.1 \mu\text{m}$) and albumin microspheres ($3.1 \mu\text{m}$) respectively did not alter the quantity or rate of microsphere uptake across the GIT. However, the quantity of albumin microspheres transported across the GIT in these studies was far less than the 39% reported by Alpar *et al.*, (1989).

The possibility of uptake and absorption of nanoparticles and microparticles by the GIT has been a controversial area, although there is now accumulated evidence that it does occur (Alpar *et al.*, 1989, Aprahamian *et al.*, 1987; Florence & Jani, 1993; Le Fèvre *et al.*, 1989; Sanders & Ashworth, 1961; Volkheimer 1977), although the mechanism of uptake still remains unclear and is discussed further in Section 1.7. The mechanism of uptake of albumin microspheres in this study remains unproven. However, once in the circulation, albumin microspheres are able to migrate to sites of inflammation, before being removed from the circulation by cells of the MPS.

Interestingly, albumin microspheres were recovered from the urine of all rats 24 hr post administration, with 5-15% of the radioactive dose being recovered in urine specimens. Jani *et al.*, (1990), after oral administration of 100 nm and $1.0 \mu\text{m}$ ^{125}I -radiolabelled polystyrene microspheres, reported 24% and 17% of the radioactive dose respectively was recovered in urine specimens. These high levels of radioactivity were thought to be due to free iodine. However, visual and SEM examination of the urine collected from rats dosed with albumin microspheres in these studies suggests the excretion of microspheres dosed orally from the body *via* the urine. Volkheimer (1977) reported the excretion of persorbed starch granules ($3-25 \mu\text{m}$) in urine of humans over an 8 hr period after ingestion of 200 gm cornstarch. Although the uptake of particulates in humans and rats may not be directly comparable, it would suggest that the radioactive doses measured in the urine samples in these experiments may not be due to free iodine.

PLATE 5.5 *Scanning Electron Micrograph Showing Albumin Microspheres Recovered From A Rat Urine Sample 24 Hours After Oral Administration Of Albumin Microspheres (Geometric Mean Diameter And σ $2.67 \pm 1.28 \mu\text{m}$)*



5.6 SUMMARY

The subcutaneous air-pouch model of inflammation was developed as a method for assessing the ability of orally and intravenously administered albumin microspheres to reach sites of inflamed tissues. The air-pouch model has been employed in previous studies for investigating the tissue distribution of non-biodegradable microspheres (Alpar *et al.*, 1989), biodegradable microspheres (Lewis *et al.*, 1992) and PMLN containing non-biodegradable microspheres (Hyde, 1990).

The degree of inflammation induced in the air-pouch tissues, after injection of the irritant carrageenan, was determined in terms of cellular infiltration and fluid accumulation within the pouch cavity, and an increase in vascular permeability in the pouch tissue. The formation of inflammation was shown to be consistent for both the acute and chronic stages, with little variation in the number of infiltrating cells and fluid volumes collected. Vascular permeability was consistently raised in both acutely and

chronically inflamed air-pouch tissues, shown by the use of radio-iodinated bovine serum albumin as a blood marker. An increase in blood vessel formation was seen by gross examination of the air-pouch tissues as the severity of induced inflammation was also increased. It was proposed that microspheres circulating within the vascular compartment may be able to migrate into inflamed tissues through gaps in the endothelial barrier and the underlying basement membrane, since these have been reported to be disrupted during the inflammatory process (Persson & Svensjö, 1985).

The ability of intravenously administered albumin microspheres (geometric mean diameter $2.67 \pm 1.28 \mu\text{m}$) to accumulate at sites of acute and chronic inflammation in the rat subcutaneous air-pouch model 4 hr post injection was investigated. The ability of Poloxamine 908-coated albumin microspheres to accumulate in inflamed tissues was compared to that of non-coated albumin microspheres. For chronically inflamed air-pouch tissues, both coated and non-coated albumin microspheres were able to accumulate in the air-pouch tissue and fluid exudates, with recovered radioactive doses of $0.9\% \text{ g}^{-1}$ and $0.17\% \text{ g}^{-1}$ in pouch tissues and 0.6% and 0.47% in whole pouch fluids respectively. Similarly for acutely inflamed air-pouch tissues, recovered radioactive doses of $0.27\% \text{ g}^{-1}$ and $0.73\% \text{ g}^{-1}$ in pouch tissues and 0.3% and 0.17% in whole pouch fluids respectively were obtained for 908-coated and non-coated albumin microspheres respectively.

The ability of orally dosed albumin microspheres (geometric mean diameter $2.67 \pm 1.28 \mu\text{m}$) to accumulate at the site of inflammation in the rat subcutaneous air-pouch model of inflammation 4 hr and 24 hr post administration was investigated. The ability of Poloxamine 908-coated albumin microspheres to accumulate in inflamed tissues was compared to that of non-coated albumin microspheres. No significant increase in the ability of neither non-coated or Poloxamine 908-coated albumin microspheres to accumulate in acutely or chronically inflamed tissues was seen 4 hr post oral administration. However, 24 hr after oral administration significant levels of both non-coated and 908-coated albumin microspheres were seen in acutely and chronically inflamed tissues. Non-coated albumin microspheres gave rise to $0.62\% \text{ g}^{-1}$ and $0.63\% \text{ g}^{-1}$ in acutely and chronically inflamed tissues respectively, with there being 0.3% and 0.78% of the radioactive dose being recovered from exudate fluids respectively. Poloxamine 908-coated albumin microspheres gave rise to similar levels of the radioactive dose in acutely and chronically inflamed tissues with $0.62\% \text{ g}^{-1}$ and $0.63\% \text{ g}^{-1}$ respectively. A significant increase in the amount of radioactive dose accumulated in the fluid exudates was seen, with 0.6% and 1.52% for acutely and chronically inflamed pouches respectively. These levels were both significantly higher than those obtained for non-coated microspheres.

From the results obtained in this study, it is suggested that albumin microspheres may escape from the vascular compartment and accumulate at sites of inflammation. Microspheres injected directly into the circulation after 4 hours and microspheres dosed orally after 24 hours are shown to accumulate in acutely and chronically inflamed tissues. The greater the degree of inflammation, the greater the degree of albumin microsphere accumulation.

CHAPTER 6

TISSUE DISTRIBUTION OF LATEX MICROSPHERES AFTER ORAL AND
INTRAVENOUS ADMINISTRATION TO RATS INDUCED WITH AN
EXPERIMENTAL MODEL OF INFLAMMATION

6. TISSUE DISTRIBUTION OF LATEX MICROSPHERES AFTER ORAL AND INTRAVENOUS ADMINISTRATION TO RATS INDUCED WITH AN EXPERIMENTAL MODEL OF INFLAMMATION

6.1. INTRODUCTION

The *in vivo* distribution of [^{125}I]-albumin microspheres was investigated and described in Chapter 5. The results suggest albumin microsphere infiltration into the subcutaneous air-pouch after oral and i.v. administration, consolidating the work of Alpar *et al.*, (1989), who reported the appearance of FITC-labelled latex particles (mean diameter 1.1 μm) in inflammatory air-pouches 4 hr after injection into the heart, and 24 hr after oral dosing, and Lewis *et al.*, (1992), who reported the accumulation of albumin microspheres (3.1 μm mean diameter) in the inflamed rat hind-paw after oral administration of particles. Due to the possible lability of the radiolabel in the prepared albumin microspheres, further studies were undertaken to give confirmation that particles could accumulate at the site of inflammation after oral and i.v. administration.

As discussed in Section 5.6, it was thought that albumin microspheres were infiltrating into inflammatory air-pouch tissues. In order to verify this, polystyrene microspheres with a mean diameter of 220 nm (± 10 nm sd) were used in similar studies. Polystyrene microspheres were chosen since their presence could be detected and quantified by gel permeation chromatography (GPC), without the problems of lability of a radiolabel and inaccurate quantitation of FITC-labelled microspheres. In this study, preliminary experiments were made, with equipment availability limiting the number of repeat experiments that were possible.

6.1.1 GEL PERMEATION CHROMATOGRAPHY

GPC is an analytical technique whereby solutes may be separated based on their molecular size (Yau *et al.*, 1979). GPC columns are tightly packed with a stationary phase. Commonly, cross-linked polystyrene, resulting from the co-polymerization of styrene and divinylbenzene is used, and the column completely filled with MP. Sample molecules dissolved in the MP are introduced onto the column *via* an injection port and permeate the stationary phase for periods of time proportional to their molecular size. Molecular size sorting takes place by the repeated exchange of solute molecules between the bulk solvent of the MP and the stagnant liquid phase, within the pores of the stationary phase. The largest molecules are eluted first, followed by those of decreasing size. The retention volume is the volume of solvent which has passed

through the system from the time of sample induction to the time of the peak maximum. Subsequent examination of the detector response verses time for a given polymer reveals its molecular size distribution. Conversion to a molecular weight distribution (MWD) is achieved by comparing the retention volumes of standard polymer fractions of narrow MWD to the retention volume of a polymer with an unknown MWD under the same experimental conditions. Once a system is standardized, the retention volume of a sample peak is directly proportional to the retention time elapsed from sample injection, and is usually referred to as such.

More elaborate data treatment involves the calculation of the polymer's molecular weight averages which gives information about chain length and the extent of chain cross-linking within a polymer. M_n is the number-average molecular weight, which is the molecular weight of the average chain length of a polymer. M_w is the weight-average molecular weight, and is the molecular weight equal to the modal molecular weight of the polymer chains. Manual calculation of M_n and M_w is accomplished by digitalization of chromatograms (Yau *et al.*, 1979). For a monodisperse system the values of M_n and M_w are the same, but usually M_w is larger than M_n , and the ratio of M_w/M_n is a measure of the spread of the molecular weight distribution. In the case of highly cross-linked polymers therefore, the value of M_w/M_n is relatively large.

Using a suitable packing material it is possible to separate molecules with molecular weights ranging from 100 to several million, with an appropriate detection and recording device to visualize the separation process. It is possible to determine unknown concentrations of the polymer of interest by interpolation from calibration plots which are statistically validated in terms of their linearity with respect to polymer concentration.

Jani *et al.*, (1990), determined the weight-average molecular weight of polystyrene microspheres by GPC analysis, enabling polystyrene in biological tissue samples analysed by GPC to be identified. Thus, with the use of calibration plots of known polystyrene concentrations, subsequent quantitation of polystyrene microsphere accumulation in the organs of rats, dosed orally with polystyrene microspheres, was possible. Tetrahydrofuran (THF) was used as the MP, being ideal for GPC due to its low viscosity and low refractive index, and polystyrene, in common with a large number of polymers, being soluble in it. Some biological molecules are also soluble in THF but, in contrast to polystyrene, are reduced to relatively small compounds by the polystyrene extraction process from tissues, and thus may be resolved from polystyrene as a separate peak on a GPC chromatogram.

6.2 MATERIALS

GPC grade THF and HPLC grade chloroform were employed. All other chemicals were of Analar or reagent grade and were purchased from appropriate suppliers as listed in Appendix I. All polystyrene extraction processes were carried out in glass conical and round-bottomed flasks and glass test tubes.

6.2.1 LATEX MICROSPHERES

Latex microspheres with a geometric mean diameter and σ_g of 220 ± 10 nm were supplied in suspension (2.5% solids) from Polyscience Inc., Northampton, UK. Before use the microspheres were washed three times in dH₂O by centrifugation to remove any surfactant remaining in the microsphere suspension, and the microspheres were then resuspended in an appropriate volume of dH₂O.

6.2.2 ANIMALS

Adult male Wistar rats (150-200 g) were purchased and maintained as described in Section 5.2.1.

6.3 INSTRUMENTATION

6.3.1 ANALYTICAL

The GPC system used was composed of a series of individual components. An Altex 110A adjustable flow rate pump was employed to pump GPC grade THF through the columns at a flow rate of 1 ml min^{-1} from a solvent reservoir. Two 300 mm x 7.5 mm (CL x ID), 10 μm mixed pore, highly cross-linked, spherical macro porous polystyrene-divinylbenzene matrix columns (PLGel, Polymer Laboratories Ltd., Shropshire, UK, 1112-6100) were set up in series and protected by a 50 mm x 7.5 mm (CL x ID), 10 μm mixed pore guard column (Polymer Laboratories Ltd., 1110-1120). Samples were introduced onto the column through a Rheodyne 7125 injector valve (Waters, CA, USA) fitted with a 100 μl sample loop. A Pye Unicam LC3 UV detector, set at a wavelength of 224 nm (sensitivity range 0.08), was connected in parallel to a Gallenkamp Euroscribe chart recorder (chart speed 2.5 mm min^{-1}) and a Hewlett Packard 3390A integrator to quantify sample detection. Effluent THF from the system was collected in a waste reservoir.

6.3.2 SUBSIDIARY

The following Sartorius electronic balances (Sartorius GMBH, Göttingen, Germany) were employed for accurate weighing purposes; an analytical A202S (4 decimal places) and a research R202D (5 decimal places). A Kerry Laboratory Sonicator was used to aid suspension of polystyrene microspheres. An Edwards Modulyo freeze-drier (BOC Ltd, Sussex, England) was employed for all freeze-drying processes and a microcentrifuge was used for centrifugation purposes at 13,000 rpm.

6.4 METHODS

6.4.1 ANAESTHESIA

Anaesthesia was induced in experimental animals for purposes of air-pouch formation and dosing regimes as described in Section 5.3.1.

6.4.2 FORMATION OF 6-DAY OLD SUBCUTANEOUS AIR-POUCHES

6-day old subcutaneous air-pouches were formed as outlined in Section 5.3.2. Chronic inflammation was induced in 6-day old subcutaneous air-pouches as outlined in Section 5.3.2.2 and used 72 hr after irritant injection.

6.4.3 POLYSTYRENE EXTRACTION PROCESS FROM TISSUE SAMPLES

Polystyrene was extracted from tissue samples based on the method developed by Jani *et al.*, (1990). Weighed sample tissues were placed in glass conical flasks, frozen (-20°C) and freeze-dried for 48 hr. The dried tissues were then immersed in liquid nitrogen, ground to a powder using a pestle and mortar, and re-freeze-dried for 24 hr. The samples were suspended in 15 ml chloroform and incubated in a 30°C shaking water bath (Tecam 5B-16, Techne Ltd., Cambridge, UK) for 48 hr to extract any polystyrene within the tissue into the organic solvent. The samples were filtered through glass wool into round-bottomed flasks, with further chloroform washing, in order to remove large tissue debris. The chloroform was evaporated to dryness using a rotary evaporator (Büchi Rotavapor R-110, Orme Scientific Ltd., Manchester, UK) and water bath at 40°C, leaving a dry film of polystyrene and biological residue deposited on the flask sides. The film and residue was dissolved typically in 2-5 ml THF, followed by filtration through a disposable, 0.5 µm pore, teflon, solvent resistant filter (Millex-SR, Millipore Waters Ltd., UK), and diluted as appropriate immediately prior

to being introduced onto the column for GPC quantitative analysis of polystyrene content.

6.4.4 CALIBRATION AND SENSITIVITY STUDIES

Column calibration was carried out using ten polystyrene standards (EasiCal[®]; Polymer Laboratories Ltd.). EasiCal[®] inert strips coated with polystyrene (about 5 mg) with molecular weight values of 6.4×10^2 , 3.25×10^3 , 9.38×10^3 , 2.87×10^4 , 6.6×10^4 , 1.58×10^5 , 3.21×10^5 , 1.03×10^6 , 3.07×10^6 , and 8.52×10^6 , were immersed in 50 ml THF for 30 min to give approximately 0.01% w/v of the polystyrene mixture. The calibration mixture was then analysed by GPC four times using the system conditions outlined in Section 6.3.1. The elution profile of a given molecular weight polystyrene standard, together with its corresponding retention time, was obtained for the GPC system employed. The relationship between the molecular weights and corresponding retention times for the calibration mixture is shown in Figure 6.1.

For determination of M_n and M_w values, 90 μg of latex microspheres (mean diameter 220 ± 10 nm sd) were dissolved in 3ml THF. 100 μl samples were injected onto the GPC system and the elution profile for each standard was recorded, from which the MWD was obtained. From the chromatogram, the M_n and M_w values were calculated by Mr J Eyles, after manual digitalization of the chromatogram, according to the method of Yau *et al.*, (1979).

In order to quantify unknown concentrations of polystyrene in biological tissue samples, a standard calibration curve was prepared. For this purpose a series of calibration solutions were made in THF, using polystyrene microsphere concentrations between 1.5-9.0 $\mu\text{g ml}^{-1}$, and analysed by GPC in triplicate. The area under the curve (from the integrator) for each of the calibration solutions was plotted against its corresponding polystyrene concentration. A typical calibration plot is shown in Figure 6.2, along with its corresponding calibration statistics shown in Table 6.1. The linearity of the plot was examined statistically by linear regression analysis to assess the validity of measuring unknown polystyrene concentrations by the same method.

Since the sample tissues undergo extensive processing to extract the polystyrene, sensitivity studies were undertaken to take into account losses which occur through vessel transfer and inefficient extraction from tissues. Thus, the efficiency of the polystyrene extraction protocol was ascertained by injecting known concentrations of microspheres into; 1) 1 g liver tissue and 2) 1 ml blood, and subjecting them to the

polystyrene extraction process, before quantifying the recovery of polystyrene by GPC analysis. A typical chromatogram showing the separation of polystyrene from liver tissue is shown in Figure 6.3. Again, calibration plots were prepared and examined statistically by linear regression analysis to assess the validity of measuring unknown polystyrene concentrations in tissues by the same method. Typical plots are shown in Figure 6.2, and the corresponding calibration statistics for these plots are shown in Table 6.1

6.5 EXPERIMENTAL

6.5.1 TISSUE DISTRIBUTION OF ORALLY DOSED LATEX MICROSPHERES

The tissue distributions of orally dosed polystyrene microspheres were compared in 2 rats, one with a 6-day old subcutaneous air-pouch with chronic inflammation, and the other with a control 6-day old air-pouch.

Animals were starved overnight before dosing. 6.51 mg (dry weight) of washed polystyrene microspheres was suspended in 1 ml dH₂O and orally administered by gavage needle. The rats were kept in metaboles, with no food but with water *ad libitum*, to collect samples of urine and faeces, before being sacrificed by CO₂ asphyxiation 24 hr after dosing. Samples of tissue from the blood, liver, lungs, spleen, kidney, stomach, urine, faeces, pouch tissue, pouch fluid and abdomen skin were removed and weighed before being subjected to the polystyrene extraction process as described in Section 6.4.3 and analysed by GPC.

6.5.2 TISSUE DISTRIBUTION OF INTRAVENOUSLY ADMINISTERED LATEX MICROSPHERES

The distribution of intravenously administered polystyrene microspheres was compared in 2 rats, one with a 6-day old subcutaneous air-pouch with chronic inflammation, and the other with a control 6-day old air-pouch.

6.51 mg (dry weight) of washed polystyrene microspheres were suspended in 250 μ l dH₂O. The microspheres were administered by tail vein injection as described in Section 5.4.3.1. 4 hr after dosing the animals were sacrificed by CO₂ asphyxiation. Samples of tissue from the blood, liver, lungs, spleen, kidney, pouch tissue, pouch fluid and abdomen skin were removed and weighed before being subjected to the polystyrene extraction process as described in Section 6.4.3 and analysed by GPC.

6.6 RESULTS AND DISCUSSION

6.6.1 CALIBRATION AND SENSITIVITY STUDIES

A polystyrene calibration mixture (EasiCal[®], Polymer Laboratories Ltd.) was used to calibrate the GPC system employed by determining the retention time on the column for each calibration standard of known mean molecular weight. Figure 6.1 is derived from plotting these retention times versus the corresponding molecular weight for each standard. These data was validated statistically by linear regression analysis and were found to have a linear correlation coefficient of 0.999. Thus, using these data, and comparing them to the chromatogram given by GPC analysis of polystyrene microspheres under the same experimental conditions, the MWD of the polystyrene which constitutes 220 nm latex microspheres (Polyscience Inc.) was obtained. Using the MWD, the values of Mn and Mw were calculated manually, after digitalization of the chromatogram, by Mr J Eyles and were found to be Mn = 160,259 and Mw = 336,223. The weight-average molecular weight of 220 nm latex microspheres was found to be in accordance with the findings of Jani *et al.*, (1990), who reported the apparent weight-average molecular weights of latex microspheres (Polyscience Inc.) with mean diameters of 100 nm and 300 nm to be 220,000 and 800,000 respectively.

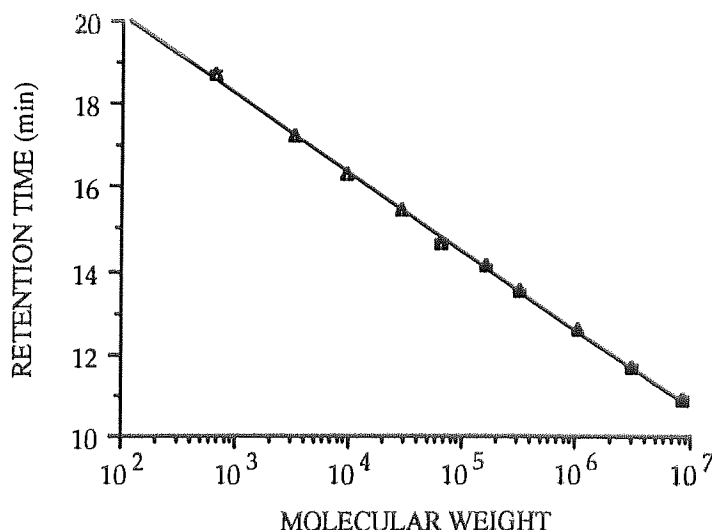
In order to determine the amount of polystyrene in tissue samples, calibration plots were made of known concentrations of polystyrene microspheres in THF, blood and liver tissues, and validated statistically in terms of their linearity with respect to polystyrene concentration. Typical calibration plots are shown in Figure 6.2, with their corresponding calibration statistics in Table 6.1. The linear regression correlation coefficients of these plots were determined to be ≥ 0.990 .

Due to the extensive processing and vessel transfer involved in the polystyrene extraction process from tissues, the efficiency of polystyrene recovery from blood and liver samples was derived from the calibration plots. Mean \pm sd percentage efficiency values of $86.1 \pm 3.2\%$ and $76.6 \pm 2.1\%$ polystyrene recovery from blood and liver tissues respectively were found when compared to THF, taken to be 100% efficiency with no extraction processes involved. Ideally, an estimate of the efficiency of polystyrene recovery from all of the tissue types analysed should be made, since from the recovery efficiencies of polystyrene from blood and liver tissues, it would appear that the more dense the tissue, the lower the recovery efficiency. However, due to equipment availability, only blood and liver tissues were used, with unknown polystyrene concentrations in blood and fluid tissues being measured using the blood

tissue calibration plot, and all other tissues being measured using the liver tissue calibration plot. Thus, the loss of polystyrene from the tissue samples in the polystyrene extraction process was accounted for when calculating unknown polystyrene concentrations in sample tissues.

A typical gel permeation chromatogram of polystyrene recovered from a liver tissue sample is shown in Figure 6.3, with the polystyrene having a retention time of 14.02 min. Biological molecules contaminating the polystyrene extraction mixture are seen to be eluted after the polystyrene, having smaller molecular weights than the polystyrene.

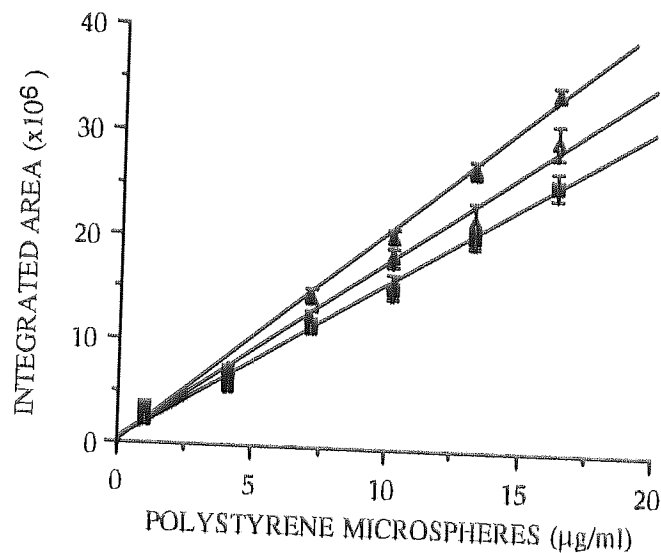
FIGURE 6.1 *Plot To Show The Relationship Between The Molecular Weight And Retention Time (min) Of A Standard Mixture Of Polystyrene (EasiCal[®], Polymer Laboratories Ltd., Shropshire, UK) With A Range Of Molecular Weights, Analysed By Gel Permeation Chromatography† Each point represents mean \pm sem (n=4). Regression correlation coefficient = 0.999*



†GPC CONDITIONS:
Column PLGel (Polymer Laboratories Ltd.,) Two x 300 x 7.5 mm CL x ID
Flow rate 1.0 ml min⁻¹; Loop size 100 μ l; Chart speed 2.5 mm min⁻¹;
Wavelength 224 nm; Sensitivity Range 0.08

MOBILE PHASE COMPOSITION:
100% THF

FIGURE 6.2 Typical Calibration Plots For Quantitative Analysis Of Latex Microspheres In THF (\blacktriangle), Blood (Δ), And Liver (\square) By Gel Permeation Chromatography†
Each point represents mean \pm sd ($n=3$)



†GPC CONDITIONS:

Column PLGel (Polymer Laboratories Ltd.,) Two x 300 x 7.5 mm CL x ID

Flow rate 1.0 ml min⁻¹; Loop size 100 µl; Chart speed 2.5 mm min⁻¹;

Wavelength 224 nm; Sensitivity Range 0.08

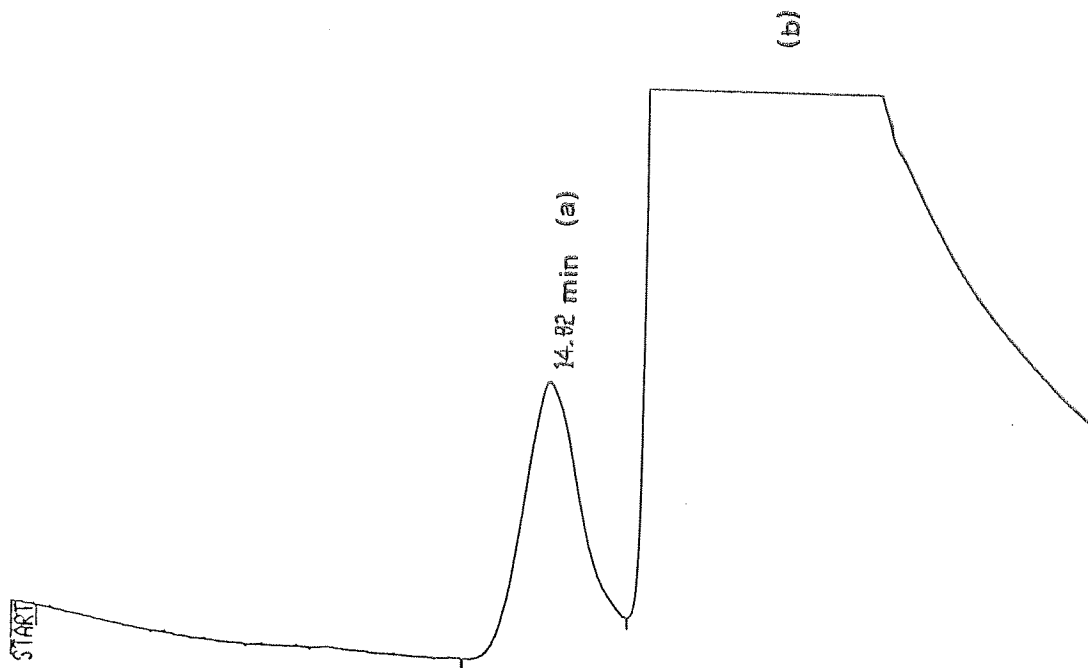
MOBILE PHASE COMPOSITION:

100% THF

TABLE 6.1 Calibration Statistics For Figure 6.2

SENSITIVITY STUDY	SLOPE	INTERCEPT	CORRELATION COEFFICIENT
THF	2.077	3.257×10^{-3}	0.993
BLOOD	1.763	1.763	0.990
LIVER	0.307	1.549	0.995

FIGURE 6.3 *Typical Gel Permeation Chromatogram For Polystyrene Extracted From A Liver Tissue Sample*



PEAK IDENTITY:
(a) polystyrene; (b) biological molecules

MOBILE PHASE COMPOSITION:
100% THF

GPC CONDITIONS:
Column PLGel (Polymer Laboratories Ltd.,) Two x 300 x 7.5 mm CL x ID
Flow rate 1.0 ml min⁻¹; Loop size 100 μl; Chart speed 2.5 mm min⁻¹;
Wavelength 224 nm; Sensitivity Range 0.08

6.6.2 TISSUE DISTRIBUTION OF ORALLY DOSED AND INTRAVENOUSLY ADMINISTERED LATEX MICROSPHERES

A preliminary study into the accumulation of non-biodegradable latex microspheres at the site of inflammation after oral and intravenous administration was undertaken to consolidate the results of similar studies using biodegradable, radiolabelled-albumin microspheres. Ideally, it would have been preferable to use latex microspheres with a mean diameter close to that of the albumin microspheres employed in similar studies (around 2.67 μm). However, only latex microspheres with a mean diameter of 220 nm were available at the time this study was undertaken.

The tissue distribution of latex microspheres 24 hr after oral dosing by gavage is shown in Table 6.2. Oral uptake of particles is evidenced by the presence of particles in the blood, liver, lung, spleen, kidney and skin tissues. These tissues are physically separate from those of the GIT, and microspheres must therefore, have penetrated the epithelial cell barrier. Total oral uptake is about 7.5% and 12.8% for the rat with the control air-pouch and the rat with the chronically inflamed air-pouch respectively. These uptake figures were higher than the 4% uptake figure obtained for albumin microspheres (2.67 μm). It has been reported by Jani *et al.*, (1990), that the uptake of 100 nm latex particles was 26%, with this figure declining as the particle size was increased, comparing to the latex and albumin microsphere oral uptake trend seen in these studies. However, the dosing schedule employed by Jani *et al.*, (1990), consisted of daily microsphere dosing by gavage over a ten day period, as opposed to the single dosing schedule employed in these studies. The percentage of microspheres in the lung of the rat with the chronic inflamed air-pouch is relatively high compared to that of the control air-pouch rat. This may be due to particle aggregation, or alternatively, by contamination of the sample during the polystyrene extraction process, although every effort was made to prevent sample contamination. The difference in the percentage of microspheres excreted in the faeces is probably due to the different extent of defecation between the two rats. A truer picture of the percentage of the dose passing through the GIT may have been made, in retrospect, by separate analysis of the small intestine, large intestine, and faeces, to give an overall picture of the microsphere distribution in the GIT 24 hr after oral dosing. The presence of latex microspheres was seen in both the control and chronically inflamed pouch tissues, with 0.03% and 1.26% of the administered dose per gram of tissue respectively. The results are expressed per gram due to the difficulty in standardizing the weight of air-pouch tissue for each rat. The accumulation of 220 nm latex particles in the chronically inflamed air-pouch is higher than that obtained for albumin microspheres (2.67 μm) of 0.6%. This may be

TABLE 6.2 *The Percentage Of The Dose Of 220 nm Latex Microspheres Distributed To Various Organs Of The Body 24 hr After Oral Dosing In The Rat Induced With An Experimental Model Of Inflammation*
For each study n = 1 rat. Results are expressed as % of administered dose recovered from the whole organ unless otherwise stated

TISSUE	CONTROL 6-DAY OLD AIR-POUCH	CHRONICALLY INFLAMED 6-DAY OLD AIR-POUCH
BLOOD	0.82	0.36
LIVER	5.26	4.97
LUNG	0.79	6.82
SPLEEN	0.22	0.24
KIDNEY	0.03	0.08
STOMACH	0.13	0.17
URINE	0.19	0.16
FAECES	72.93	39.27
POUCH FLUID	no fluid accumulation	0.01
POUCH TISSUE	0.03 g ⁻¹	1.26 g ⁻¹
ABDOMEN TISSUE	0.48 g ⁻¹	0.90 g ⁻¹

due to the smaller size of the latex microspheres being able to escape from the damaged endothelium and basement membrane to a greater extent than the larger albumin microspheres.

Thus, a tentative conclusion from these data would suggest that latex microspheres (220 nm) may be accumulated at the site of inflammation after oral dosing. GPC analysis of abdomen skin tissues also shows the presence of microspheres in skin tissues. Further repeat experiments need to be undertaken to clarify whether the microspheres are accumulating at the inflamed sites.

Table 6.3 shows the tissue distribution of intravenously administered latex microspheres 4 hr after dosing. In both the control air-pouch rat and the chronically inflamed air-pouch rat, the latex microspheres were removed from the circulation by the Kupffer cells of the liver, with 39.15% and 36.99% of the administered dose respectively being recovered from this tissue. 7.04% and 9.04% of the microspheres dosed were accumulated in the lungs of the control air-pouch rat and the chronically inflamed air-pouch rat respectively. This suggests aggregation of the particles prior to

dosing, since relatively small particles of 220 nm mean diameter would be expected to remain in the blood during the mechanical filtering process of the lung. The presence of microspheres in the pouch fluid suggests the passage of microspheres through the capillary endothelium of the blood vessels into the pouch cavity. Microspheres are also seen in the tissue of both the control and the inflamed air-pouches. Again, it is hard to determine the total percentage of the microsphere dose in these tissues, although more of the dose appears to be accumulating in the pouch with chronic inflammation. More experiments need to be undertaken to assess whether there is significant microsphere accumulation at the inflamed sites.

TABLE 6.3 *The Percentage Of The Dose Of 220 nm Latex Microspheres Distributed To Various Organs Of The Body 4 hr After I.V. Administration For each study n = 1 rat. Results are expressed as % of administered dose recovered from the whole organ unless otherwise stated*

TISSUE	CONTROL 6-DAY OLD AIR-POUCH	CHRONIC 6-DAY OLD AIR-POUCH
LIVER	39.15	36.99
LUNG	7.04	9.04
SPLEEN	0.53	3.12
KIDNEY	none detected	none detected
BLOOD	0.42	1.84
POUCH FLUID	no fluid accumulation	0.77
POUCH TISSUE	0.21 g ⁻¹	0.37 g ⁻¹
ABDOMEN TISSUE	0.13 g ⁻¹	0.18 g ⁻¹

More extensive studies are necessary to confirm the accumulation of latex microspheres at the site of inflammation, and latex microspheres around 2.5 µm are necessary to confirm the radiolabelled albumin microsphere studies described in Chapter 5. It would be interesting to determine the percentage of recovery of latex microspheres from air-pouch tissue, since this thick, granulomatous tissue is hard to grind to a powder for polystyrene extraction purposes. It would be expected that the percentage recovery would be lower than the 76.6% recovery of polystyrene from liver tissues, and hence measurement of latex microspheres in pouch tissues may be lower the true value.

6.7 SUMMARY

These studies culminated in the standardization of a GPC assay system which may be used for the quantitation of latex microsphere tissue distribution studies after administration to rats. The efficiency of polystyrene extraction from the latex microsphere-spiked blood and liver tissues were 86.1% and 76.6% respectively. This compared favourably to the work of Jani *et al.*, (1990), who reported a mean value of 74% polystyrene recovery from spiked liver tissues.

The accumulation of 220 nm latex microspheres at the site of chronic inflammation was investigated 4 hours after intravenous administration and 24 hours after oral administration of particles. Intravenous administration of particles gave rise to the accumulation of 0.37% g⁻¹ of the injected dose in chronically inflamed air-pouch tissue and oral administration by gavage needle gave rise to the accumulation of 1.26% g⁻¹ of the administered dose in chronically inflamed air-pouch tissue. Thus, it is suggested that particles may be absorbed across the GIT, and once in the circulation may migrate out of the vasculature at sites of chronic inflammation.

CHAPTER 7

USE OF THE CARRAGEENAN-INDUCED RAT HIND-PAW MODEL OF INFLAMMATION TO DETERMINE THE THERAPEUTIC USE OF STEROID- INCORPORATED ALBUMIN MICROSPHERES

7. USE OF THE CARRAGEENAN-INDUCED RAT HIND-PAW MODEL OF INFLAMMATION TO DETERMINE THE THERAPEUTIC EFFECTIVENESS OF STEROID-INCORPORATED ALBUMIN MICROSPHERES

7.1 INTRODUCTION

Lewis *et al.*, (1992), have previously reported the use of carrageenan-induced hind-paw inflammation in the rat as a method of assessing the anti-inflammatory action of orally administered, steroid-incorporated, albumin microspheres. The following studies were undertaken to consolidate the results of Lewis *et al.*, (1992), in terms of the ability of orally administered, steroid-incorporated, albumin microspheres to reduce oedema formation, and to enhance the preliminary study made by Lewis *et al.*, (1992), on the ability of steroid-incorporated albumin microspheres, injected intra-peritoneally (i.p.), to reduce oedema formation in the rat hind-paw model of inflammation.

7.1.1 CARRAGEENAN-INDUCED OEDEMA IN THE RAT HIND-PAW FOR TESTING THE EFFICACY OF ANTI-INFLAMMATORY DRUGS

Elevation of the permeability to macromolecules in local vascular networks is recognised as one of the most prominent features in the inflammatory process, with the clinical symptoms of tissue oedema and fluid accumulation in body cavities resulting (Persson & Svensjö, 1985). Therefore, the anti-inflammatory activity of drugs has commonly been evaluated on the basis of their suppressive action against this phenomenon. Among a number of methods to date, carrageenan-induced hind-paw oedema of the rat, developed by Winter *et al.*, (1962), is one of the most commonly employed techniques used in assessment of the anti-exudate activity of drugs.

Subplantar injection of carrageenan into the hind-paw of a rat produces profound swelling of the paw within a few hours, with the maximal vascular response appearing between 3-6 hours post injection, the mechanism of which is reviewed by Di Rosa (1972). Foot volume is recorded immediately after irritant injection and at a pre-determined time period post injection, the difference in paw volume being recorded as the 'volume of oedema formation'. This method was first used to measure the anti-inflammatory activity of indomethacin (Winter *et al.*, 1963) and, with slight modifications, has become a popular test for steroidal and non-steroidal anti-inflammatory compounds (Ohno *et al.*, 1972; Tsurufuji *et al.*, 1978). It should be remembered, however, that this type of carrageenan-induced inflammation, even

though it is an excellent model for acute inflammation, may not be comparable to the inflammation of synovia in RA, since the synovitis is a specialized type of chronic granulomatous tissue. Also, it should be noted that anti-inflammatory drugs are not given to patients before clinical symptoms of the disease are presented, but are used when the disease process has already been established.

7.2 MATERIALS

All chemicals were of Analar or reagent grade and were purchased from appropriate suppliers as listed in Appendix I. All solutions and suspensions were made with dH₂O unless otherwise stated.

7.2.1 ALBUMIN MICROSPHERES

Heat-denatured albumin microspheres were prepared as outlined in Section 2.4.1.2, having a geometric mean diameter and σ_g of $2.45 \pm 1.82 \mu\text{m}$.

7.2.2 STEROID-INCORPORATED ALBUMIN MICROSPHERES

Steroid-incorporated heat-denatured albumin microspheres, prepared by the enzymatic conversion of H-21-P to HC method, as outlined in Section 4.4.2, were employed, with a geometric mean diameter and σ_g of $2.47 \pm 1.75 \mu\text{m}$, and a steroid loading of between 12.5-15% w/w.

7.2.3 ANIMALS

Adult male Wistar rats (150-200 g) were purchased and maintained as described in Section 5.2.1.

7.3 INSTRUMENTATION

7.3.1 ANALYTICAL

A reservoir of mercury was contained in a glass cylinder 25 mm diameter and 60 mm deep. The mercury column was connected to a Statham pressure transducer (P23BB, 0-5 cm Hg). The output from the transducer was led, through a Statham control unit, to a Washington chart recorder. Readings were calibrated in terms of ml displacement of mercury; immersion of an object with a volume of 1 ml produced a peak on the chart

recorder with an average height of 10.0 ± 0.03 mm ($n=25$). The method was reproducible and provided a rapid measure of foot paw volume.

7.3.2 SUBSIDIARY

Boyle's apparatus was used to deliver gases for anaesthetic purposes. The following Sartorius electronic balances (Sartorius GmbH, Göttingen, Germany) were employed for accurate weighing purposes; an analytical A200S (4 decimal places) and a research R200D (5 decimal places).

7.4 METHODS

7.4.1 ANAESTHESIA

Halothane anaesthesia was induced in the experimental animals as outlined in Section 5.3.1. The animals were anaesthetized prior to carrageenan injection, and again prior to hind-paw measurements.

7.4.2 CARRAGEENAN-INDUCED HIND-PAW INFLAMMATION IN THE RAT

50 μ l sterile 0.5% w/v carrageenan in physiological saline solution was injected subplantar into the right hind-paw of the rat. 50 μ l physiological saline was similarly injected into the left hind-paw as a control measure.

7.4.3 MEASUREMENT OF OEDEMA FORMATION IN THE RAT HIND-PAW MODEL OF INFLAMMATION

The method employed for induction and measurement of hind-paw inflammation was based on that reported by Lewis *et al.*, (1992), with a single modification. Hind-paw volume measurements were recorded 5 hr after carrageenan injection, rather than the reported 3 hr. Di Rosa, (1972), observed a well-defined and highly developed cellular exudate at 4 hr post carrageenan injection, with an increase in the number of cells by 6 hr. Due to working time restraints, 5 hr hind-paw measurements were taken instead of 6 hr measurements, since these were considered to be a better compromise than 3 hr measurements, allowing further development of the inflammatory response to carrageenan.

Immediately after control and irritant injections into the right and left hind-paw of a rat respectively, the hind-paws of the anaesthetized rat were immersed, in turn, into the mercury bath exactly to an ink mark on the skin drawn just below the fur line. This was repeated three times for each hind-paw to record an average initial hind-paw peak height reading. After irritant and control injections, inflammation was allowed to develop for 5 hr before the rats were re-anaesthetized and the hind-paw volumes measured in the same manner. The increase in hind-paw volume was calculated as the percentage increase in hind-paw volume 5 hr after irritant injection.

7.5 EXPERIMENTAL

7.5.1 STUDY OF THE ABILITY OF ORALLY DOSED STEROID-INCORPORATED ALBUMIN MICROSPHERES TO REDUCE OEDEMA FORMATION IN THE CARRAGEENAN-INDUCED RAT HIND-PAW MODEL OF INFLAMMATION

The oral steroid dose of 17.5 mg kg^{-1} used in these experiments was based on the work of Winter *et al.*, (1962), and Lewis *et al.*, (1992). The former group showed that an oral dose of 18 mg kg^{-1} inhibited carrageenan-induced oedema formation by 61%, compared to a reduction of 35% and 26% for doses of 6 mg and 2 mg HC respectively. Lewis *et al.*, (1992), used a dose of 17.5 mg kg^{-1} HC for i.p. studies to investigate the therapeutic effectiveness of this route of steroid-incorporated albumin microsphere administration. Therefore, a steroid dose of 17.5 mg kg^{-1} was utilized to compare the therapeutic effectiveness of oral and i.p. routes of steroid-incorporated albumin microsphere administration. Plain albumin microspheres were administered alone to account for possible anti-inflammatory action *via* a counter-irritant effect (Laden *et al.*, 1958). To comply with consistency, free drug was administered with an equivalent weight of plain microspheres.

12 male Wistar rats were divided into 4 sets of 3 animals and starved overnight, before their initial hind-paw volumes were measured, as outlined in section 7.4.3. The following oral dosing schedule was followed;

Set 1; An equivalent weight of plain albumin microspheres to steroid-incorporated microspheres (typically between 12-15 mg microspheres), were suspended in 1 ml PBS, vortexed thoroughly and administered by gavage needle.

Set 2; HC was weighed to give an equivalent dose to 17.5 mg kg^{-1} and suspended in 1 ml PBS. An equivalent weight of plain albumin microspheres to steroid-incorporated

microspheres were suspended in the prepared drug suspension, vortexed thoroughly and administered by gavage needle.

Set 3: H-21-P was weighed to give an equivalent dose to 17.5 mg kg^{-1} and dissolved in 1 ml PBS. An equivalent weight of plain albumin microspheres to steroid-incorporated microspheres were suspended in the prepared drug solution, vortexed thoroughly and administered by gavage needle.

Set 4: Steroid-incorporated albumin microspheres were weighed to give a steroid dose equivalent to 17.5 mg kg^{-1} . Immediately prior to dosing, the microspheres were suspended in 1 ml PBS, vortexed thoroughly and administered by gavage needle.

60 min after dosing, hind-paw inflammation was induced as described in Section 7.4.2. 5 hr after irritant injection, final hind-paw volumes were measured.

The results were expressed in terms of the % increase in hind-paw volume \pm sd (n=3 rats per group). The % increase in hind-paw volume found after the administration of plain albumin microspheres was taken as 100% oedema formation, and the % reduction in hind-paw oedema formation calculated from this figure. The results are shown in Section 7.6.1.

7.5.2 STUDY OF THE ABILITY OF INTRA-PERITONEALLY ADMINISTERED STEROID-INCORPORATED ALBUMIN MICROSPHERES TO REDUCE OEDEMA FORMATION IN THE CARRAGEENAN-INDUCED RAT HIND-PAW MODEL OF INFLAMMATION

12 male Wistar rats were divided into 4 sets of 3 animals and their initial hind-paw volumes measured. The dosing schedule employed was the same as in Section 7.5.1 with the single modification that each dose was suspended in 0.5 ml PBS and administered i.p.

60 min after dosing, hind-paw inflammation was induced as described in Section 7.4.2. 5 hr after irritant injection, final hind-paw volumes were measured as outlined in Section 7.4.3. The results were expressed in terms of the % reduction in the increase of hind-paw volume after carrageenan injection, and are shown in Section 7.6.2.

7.6 RESULTS AND DISCUSSION

7.6.1 DETERMINATION OF THE ABILITY OF ORALLY DOSED STEROID-INCORPORATED ALBUMIN MICROSPHERES TO REDUCE OEDEMA FORMATION IN THE CARRAGEENAN-INDUCED RAT HIND-PAW MODEL OF INFLAMMATION

The ability of orally administered, steroid-incorporated albumin microspheres to inhibit oedema formation in the inflamed rat hind-paw induced with 50 µl 0.5% w/v carrageenan in physiological saline solution was used as a measure of their therapeutic effectiveness (Table 7.1). When compared to control plain albumin microspheres, equivalent doses of free HC, free H-21-P, and HC/H-21-P-incorporated albumin microspheres were all able to significantly reduce oedema formation, with a reduction of 57.6%, 78.2% and 74.6% in oedema formation respectively. However, no significant difference in the ability of steroid-incorporated albumin microspheres to inhibit oedema formation was observed when compared to equivalent doses of either free HC or H-21-P.

TABLE 7.1 *The Percentage Increase In Hind-Paw Volume And The Percentage Reduction Of Oedema Formation In The Carrageenan-Induced Rat Hind-Paw By Orally Administered, Steroid-Incorporated, Albumin Microspheres (17.5 mg kg⁻¹) Measured 5 Hours After Irritant Injection Each figure represents mean ± sd (n=3 rats) ¹Significantly different from control plain albumin microspheres *P<0.005*

DOSING REGIME	% INCREASE IN HIND-PAW VOLUME	% REDUCTION IN OEDEMA FORMATION
PLAIN ALBUMIN MICROSPHERES	59.5 ± 7.7	-
HC SUSPENSION + PLAIN MICROSPHERES	25.2 ± 4.7	¹ *57.6%
HCP SOLUTION + PLAIN MICROSPHERES	12.9 ± 9.7	¹ *78.2%
STEROID INCORPORATED MICROSPHERES	15.1 ± 9.2	¹ *74.6%

7.6.2 DETERMINATION OF THE ABILITY OF INTRA-PERITONEALLY ADMINISTERED STEROID-INCORPORATED ALBUMIN MICROSPHERES TO REDUCE OEDEMA FORMATION IN THE CARRAGEENAN-INDUCED RAT HIND-PAW MODEL OF INFLAMMATION

The ability of intra-peritoneally administered, steroid-incorporated albumin microspheres to inhibit oedema formation in the inflamed rat hind-paw induced with 50 μ l 0.5% w/v carrageenan in physiological saline solution was used as a measure of their therapeutic effectiveness (Table 7.2). When compared to control plain albumin microspheres, free HC, free H-21-P, and HC/H-21-P-incorporated albumin microspheres were all able to significantly reduce oedema formation, with a reduction of 42.5%, 53.6% and 40.9% in oedema formation respectively. However, no significant difference in the ability of steroid-incorporated albumin microspheres to inhibit oedema formation was seen when compared to equivalent doses of either free HC or H-21-P.

TABLE 7.2 *The Percentage Increase In Hind-Paw Volume And The Percentage Reduction Of Oedema Formation In The Carrageenan-Induced Rat Hind-Paw By Intra-Peritoneally Administered, Steroid-Incorporated, Albumin Microspheres (17.5 mg kg⁻¹) Measured 5 Hours After Irritant Injection*
*Each figure represents mean \pm sd (n=3 rats) ¹Significantly different from control plain albumin microspheres *P<0.005 **P<0.01*

DOSING REGIME	% INCREASE IN HIND-PAW VOLUME	% REDUCTION IN OEDEMA FORMATION
PLAIN ALBUMIN MICROSPHERES	45.2 \pm 9.1	-
HC SUSPENSION + PLAIN MICROSPHERES	26.0 \pm 11.4	¹ **42.5%
HCP SOLUTION+ PLAIN MICROSPHERES	21.0 \pm 3.0	¹ *53.6%
STEROID INCORPORATED MICROSPHERES	23.3 \pm 10.7	¹ **40.9%

The % increase in hind-paw volume for plain microsphere controls in each group of experiments shows a difference, with an increase of 59.5% and 45.2% for orally and intra-peritoneally dosed plain microspheres respectively. The difference in % increase in paw volume between controls for the two sets of data may be due to the two different carrageenan solutions employed, and not due to an enhanced inflammatory effect of the microspheres administered *via* the oral route. However, since the data was normalized with respect to the plain albumin microsphere controls, it was possible to compare the effectiveness of the steroid formulations administered *via* the two different routes.

Interestingly, the ability of orally dosed steroid, both the free doses and the steroid-incorporated albumin microspheres, appeared to inhibit oedema formation to a greater extent than equivalent steroid dosage forms administered intra-peritoneally. This may be due to a difference in the time taken for steroid to be absorbed into the blood stream after administration *via* the oral and intra-peritoneal routes. HC is readily absorbed across the GIT in about 90 minutes (George & Kirwan, 1990). No data has been found for HC absorption *via* the intra-peritoneal route.

There appeared to be no significant benefit in administering steroid-incorporated albumin microspheres, as opposed to free steroid, *via* either route of administration. The *in vitro* drug release profiles show steroid is quickly released from the prepared albumin microspheres, with all the incorporated steroid being released within the first 10 minutes of dissolution. Therefore, in these experiments, in which the steroid-incorporated microspheres were suspended in PBS prior to administration, it is expected that the drug will be released from the microspheres prior to oral or i.p. administration. Equivalent doses of steroid were thus administered with similar bioavailabilities, giving rise to similar degrees of inhibition of oedema formation.

In contrast to the results given by this study, Lewis *et al.*, (1992), reported that steroid-incorporated albumin microspheres were more effective than free steroid in reducing oedema formation in carrageenan-induced inflammation of the rat hind-paw. However, in these experiments, free HC (17.5 mg kg^{-1}) was administered intra-peritoneally whereas steroid-incorporated albumin microspheres (17.5 mg kg^{-1}) were orally administered. The results showed a percentage inhibition of $67 \pm 6.2\%$ and $72.1 \pm 25.6\%$ respectively for free intra-peritoneally administered HC and orally administered steroid-incorporated microspheres respectively. The results shown in Tables 7.1 and 7.2 would suggest that the steroid-incorporated albumin microspheres administered orally were not better in reducing oedema formation than the equivalent

administered orally were not better in reducing oedema formation than the equivalent dose of free steroid also administered orally, but would appear better when compared to an equivalent dose of intra-peritoneally administered free steroid.

The degree of oedema formation in each animal was variable. Some animals showed very little oedema formation in response to the irritant, whereas others showed an acute response with a large amount of swelling. This variation between each animal is reflected in the large standard deviation statistics. It would, therefore, have been preferable to have larger numbers of rats per group, to try to improve consistency. The assay system employed was shown to give consistent measurements of known volumes. To increase accuracy in the measurement of hind-paw volumes, the rats were lightly anaesthetized. This made it possible to accurately measure the hind-paws by steadily dipping them in the mercury bath up to the ink marks.

On reflection, the carrageenan-induced oedema in the rat hind-paw as an assay system for testing the efficacy of steroid-incorporated albumin microspheres has many limitations. The assay is employed as a measure of the ability of drugs to respond to acute inflammation, whereas it would be preferable to test the steroid-incorporated microspheres therapeutic effectiveness against chronic inflammation, as seen in the rheumatoid synovium. If the steroid-incorporated albumin microspheres had shown a slower drug release, then the duration of this assay would not have been long enough to see whether this was beneficial compared to equivalent free steroid doses. Finally, the steroid doses were administered prior to the inducement of inflammation, a situation which is not seen in RA patients, where the disease is well established before there are severe enough clinical symptoms for the patient to receive drug therapy of this kind. A more suitable assessment of the anti-inflammatory effect of steroid-incorporated albumin microsphere preparations would be made using animal models of chronic inflammation, such as antigen-induced arthritis or the subcutaneous pouch granuloma model of inflammation.

7.7 SUMMARY

Carrageenan-induced oedema of the rat hind-paw was used as an animal model of inflammation. This animal model was employed to test the effectiveness of steroid-incorporated albumin microspheres to reduce oedema formation when compared to equivalent free steroid doses. Two different routes of steroid administration were investigated, namely oral dosing by gavage needle and intra-peritoneal injection. The results show steroid-incorporated albumin microspheres administered by both routes

were as effective as equivalent free steroid control doses in reducing carrageenan-induced oedema formation in the rat hind-paw, but did not show an enhanced effect. It would appear that equivalent steroid doses, when administered orally as opposed to intra-peritoneally, were more effective in reducing oedema formation for the assay protocol employed in these studies.

CHAPTER 8

CONCLUDING REMARKS

8. CONCLUDING REMARKS

RA is a chronic, systemic disorder that is dominated by the erosion of articular joint tissues, often leading to serious patient disability. Despite extensive research, the etiologic agent remains unknown to this day. RA patients present clinically heterogenic symptoms, leading to delay and confusion in diagnosis of the disease. Due to the clinical heterogeneity of RA, it is not possible to describe a therapeutic regime which is applicable to all RA patients, and the treatment of RA remains one of the most challenging problems in clinical medicine today. The major treatment goals are aimed initially at reducing the patient's pain and discomfort, and controlling the inflammation within the diseased joint(s), followed by regaining joint(s) function and mobility, and finally retarding the progression of joint(s) destruction. However, no single treatment is available to achieve these therapeutic goals, and generally the treatment is in a pyramid format, beginning with the use of NSAIDs to reduce pain and inflammation, followed by DMARDs, cytotoxic agents and corticosteroids to retard joint destruction. However, the use of these drugs is complicated by their adverse side effects, and methods to reduce their toxicity are currently being developed in the form of drug delivery systems.

For the treatment of RA, there are predominately two forms of drug delivery systems which have been investigated, namely locally injected, sustained release devices, and parenterally and non-parenterally administered site-specific drug delivery systems. Various materials have been used in the manufacture of both types of delivery systems including liposomes, and particulates composed of both natural and synthetic polymers. Publications have advocated the biocompatibility and biodegradability of albumin microspheres *in vivo*, and these delivery systems have been loaded with anti-inflammatory drugs. This project was designed to study the use of microspheres to target steroidal drugs to the site of inflammation by the oral and intravenous routes.

The production of albumin microspheres of a particle size convenient for oral and intravenous delivery ($<3 \mu\text{m}$) was carried out using both thermal-denaturation and chemical cross-linking methods of microsphere stabilization, resulting in spherical and well formed microspheres. Once parameters such as the protein concentration, and volume ratios of the liquid phases were chosen, the microsphere size could be adjusted by the speed and length of the homogenization process.

An HPLC assay method was developed to precisely and simultaneously quantitate the loading and *in vitro* release of steroid incorporated in albumin and PLA microspheres.

In addition, the use of HPLC methods showed the stability of the steroids during both the albumin microsphere manufacturing process, where the drug is exposed to temperatures exceeding 100°C, and the PLA manufacturing process where the drug is exposed to organic solvents.

For a drug delivery system to be effective in alleviating disease symptoms, it is imperative to attain the minimum effective concentration of medication at the target site, and an increase in drug entrapment in the microspheres would reduce the amount of carrier required to deliver a specified dose of drug. Therefore it is essential to design microspheres with high levels of drug entrapment. Three methods were investigated to load HC in albumin microspheres and, using the freely water-soluble hydrocortisone disodium salt, H-21-P, followed by the use of the enzyme alkaline phosphatase to convert H-21-P to HC, it was possible to achieve high steroid-incorporation levels around 30% (w/w). However, only about 1-2% of the steroid loaded was HC, but with favourable drug release over a period of days, this total steroid loading would still give rise to an attractive drug delivery system, since the H-21-P would be converted to HC *in vivo* by phosphatases within the body tissues. Similar HC loadings of 1.4% (w/w) were achieved by the direct incorporation of HC method, and these results are comparable to those of other workers for similar steroids incorporated into albumin microspheres (Saunders *et al.*, 1991). The adsorption of HC onto the surface of empty microspheres gave a relatively low steroid loading of <0.5% (w/w), when compared to that achieved by other workers (6-9% w/w, Lewis *et al.*, 1992). Both of these latter methods achieved steroid loading levels that were too low for viable drug delivery systems.

In vitro release of steroid from albumin microspheres loaded by the enzymatic conversion of H-21-P to HC method showed >95% of the incorporated drug was released within the first 2 hours of dissolution under sink conditions. In order to prolong the release of entrapped drug, the steroid:protein ratio was increased, along with the length and temperature of the stabilization process. The alteration in steroid:protein ratio and increase in microsphere denaturation temperature, had little effect on prolonging drug release. An increase in the length of denaturation gave rise to >84% of the drug being released after the first 2 hours. These results are in contrast to those found by other workers who showed the release of drugs from albumin microspheres may be prolonged by increasing the length and temperature of thermal denaturation (Burgess *et al.*, 1987, Gupta *et al.*, 1989a). The biphasic release pattern of steroid seen in these studies is characteristic for albumin microspheres. Such high initial bursts of drug from steroid-incorporated albumin microspheres would lead to

premature release of steroid *in vivo*, with the pharmacological effects of the drug being comparable to that of free drug. This was shown using the rat hind-paw model of inflammation. Equivalent doses of both free steroid, and steroid-incorporated in albumin microspheres dosed both orally and intra-peritoneally, showed similar abilities to inhibit oedema formation induced in the rat hind-paw.

PLA microspheres (<3 μm) were manufactured by the o/w single emulsion version of the solvent evaporation method, giving rise to spherical and well-formed microspheres. HC was incorporated into the microspheres by addition of drug to the internal phase, giving rise to a drug incorporation of 1.2% (w/w). By using a co-solvent system in the internal phase to increase HC solubility, and by saturating the aqueous phase with HC to prevent HC loss from formed microspheres, a relatively high loading of 10.6% (w/w) was achieved. This compares favourably to the work of Mahato *et al.*, (1992), who, using a similar co-solvent system for the loading of HC into PLGA microspheres achieved a loading of 7.5% (w/w). However, *in vitro* release studies again showed >94% of the incorporated drug was released within the first two hours of dissolution and hence this formulation was not suitable as a drug delivery system for targeting to the site of inflamed tissues.

Ideally, oral dosing of site-specific drug delivery systems would be preferable. However, there exists many anatomical barriers which the drug delivery system must overcome for this route of delivery to be successful. The uptake of particulates across the GIT still remains a controversial area, although a body of evidence is now accumulating in the literature supporting its occurrence, although the mechanism(s) of uptake and size of particulates transported across the GIT still remains in question. The subcutaneous air-pouch was successfully developed as a model of both acute and chronic inflammation for testing the ability of albumin microspheres to reach inflamed tissues after intravenous and oral dosing.

Four hours after intravenous dosing, albumin microspheres were traced in both acutely and chronically inflamed tissues, with accumulation being enhanced in chronically inflamed tissues. The results indicate that albumin microspheres (2.67 μm) were able to migrate from the vascular compartment through disrupted endothelium and basement membranes into the air-pouch tissues and fluid exudates. Other workers have also advocated the accumulation of particulates at sites of inflammation using different types and sizes of particulates along with different models of inflammation (Alpar *et al.*, 1989; Illum *et al.*, 1989, Love *et al.*, 1989). From the investigations made by these workers, it was found that particulate size is one of the major factors affecting

accumulation of particulates at inflamed sites. The smaller the mean particle diameter, the greater the microsphere accumulation. Since only about 30% of the albumin microspheres administered in these studies were $\leq 1 \mu\text{m}$, it is plausible that greater albumin microsphere accumulation at the site of inflammation may be possible if the mean particle size was decreased.

No increase in albumin microsphere accumulation in inflamed tissues was seen 4 hours after oral dosing by gavage. However, accumulation of microspheres was seen in acutely and chronically inflamed tissues 24 hours after dosing. Since the uptake of microspheres across the GIT into the circulation has been reported within 5 min of dosing (Lewis *et al.*, 1992), the accumulation of particles at 4 hours as well as 24 hours would be expected, with the results suggesting possible lability of the radiolabel and breakdown of the albumin microspheres.

Preliminary, confirmatory studies using non-biodegradable latex microspheres ($0.22 \mu\text{m}$) and GPC analysis of particle accumulation in inflamed tissues, showed accumulation of latex microspheres in inflamed tissues 4 hours after intravenous administration and 24 hours after oral dosing.

The studies on the distribution of albumin microspheres to the site of acute and chronic inflammation are not conclusive, and more extensive tissue distribution studies need to be undertaken. Uptake studies using latex microspheres of a comparable size to albumin microspheres would have enhanced the viability of the radiolabelled albumin microsphere work. However, this study suggests the uptake of albumin microspheres ($2.67 \mu\text{m}$) across the GIT and into the circulation, where particles may migrate from the vasculature at inflamed sites and accumulate in locally inflamed tissues. It must be remembered that these studies are carried out in animal models of induced inflammation, and these cannot be directly compared to the chronic inflammation seen in RA patients. The long term pain and disability of patients suffering from RA warrants further investigations into methods of improving drug therapy of this disease, including the use of particulate drug delivery systems.

CHAPTER 9

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CHAPTER 10

APPENDICES

APPENDIX I

Agar	Sigma Chemical Co. Ltd., Poole, UK
Bovine Serum Albumin Fraction V	Sigma Chemical Co. Ltd., Poole, UK
Carrageenan λ Fraction	Sigma Chemical Co. Ltd., Poole, UK
Cortisone	Sigma Chemical Co. Ltd., Poole, UK
Diethylamine	BDH Chemicals Ltd., Poole, UK
Diethylether	Fisons Scientific Equipment, Loughborough, UK
Eosin	Sigma Diagnostics
Ethanol	Fisons Scientific Equipment, Loughborough, UK
Glutaraldehyde	BDH Biomedicals Ltd., Poole, UK
Glycerol	Fisons Scientific Equipment, Loughborough, UK
Haematoxylin	Sigma Diagnostics
Hydrocortisone	Sigma Chemical Co. Ltd., Poole, UK
Hydrocortisone-21-Phosphate	Sigma Chemical Co. Ltd., Poole, UK
Methanol (HPLC grade)	Fisons Scientific Equipment, Loughborough, UK
Methyl-paraben	Sigma Chemical Co. Ltd., Poole, UK
n-Heptane	Fisons Scientific Equipment, Loughborough, UK
Olive Oil	Sigma Chemical Co. Ltd., Poole, UK
Orthophosphoric Acid	Fisons Scientific Equipment, Loughborough, UK
Polyvinylalcohol	BDH Chemicals Ltd., Poole, UK
Prednisolone	Sigma Chemical Co. Ltd., Poole, UK
Propylene glycol	Sigma Chemical Co. Ltd., Poole, UK
Propyl Paraben	Sigma Chemical Co. Ltd., Poole, UK
Tetrahydrofuran (GPC grade)	Aldrich Chemical Co. Ltd., Gillingham, UK
Water (HPLC grade)	Fisons Scientific Equipment, Loughborough, UK

APPENDIX II

PHOSPHATE BUFFERED SALINE pH 7.4

Dissolve 2.38 g of disodium hydrogen orthophosphate, 0.19 g of potassium dihydrogen orthophosphate and 8.0 g of sodium chloride in sufficient water to produce 1000 ml. Adjust the pH immediately before use if necessary.

To prepare quarter strength PBS (37mM), add one part PBS to three parts dH₂O.

PHOSPHATE BUFFER pH 7.0, 0.067M

SOLUTION A

Dissolve 0.908 g of potassium dihydrogen orthophosphate in sufficient water to produce 100 ml.

SOLUTION B

Dissolve 2.38 g of disodium hydrogen orthophosphate in sufficient water to produce 100 ml.

Mix 38.9 ml of solution A with 61.1 ml solution B.

PHOSPHATE BUFFER pH 7.0, 0.0067M

Dilute one volume of 0.067M mixed phosphate bufer pH 7.0 to 10 volumes with water.

APPENDIX III

TISSUE EMBEDDING AND SECTIONING

SPECIMEN FIXATION

Tissues were cut into 1 mm sections and fixed in 10% v/v formaldehyde saline solution for 48 hr.

SPECIMEN DEHYDRATION

The fixed specimens were dehydrated for 24 hr in absolute alcohol with periodic changes of the alcohol solution.

SPECIMEN EMBEDDING

A glycol methacrylate solution (Solution A) was prepared as follows:

Hydroxyethyl methacrylate	90 ml
Butoxyethanol	10 ml
Polyethylene glycol 400	2 ml
Benzoyl peroxide	1.5 g

The fixed and dehydrated specimens were soaked in this solution for 24 hr before being transferred into fresh Solution A and soaked for a further 2 hr.

The polymerizer solution (Solution B) was prepared immediately before use as follows:

Solution A	10 ml
Dimethylanylne	1 drop

Solution B was poured into small plastic embedding moulds (Agar Scientific Limited, Essex, England) up to the first lip. Each specimen was immersed into a mould and the mould filled to the brim with Solution B, ensuring no air bubbles were formed. The moulds were left for 2 hr to cure before being removed from the plastic moulding.

SPECIMEN SECTIONING

Using an ultramicrotome, the tissue specimens were cut into 5 μ m sections, floated on water and placed on glass microscope slides. The sections were left to dry on a hot plate before being stained with an appropriate stain and visualized by light microscopy.

APPENDIX IV

HAEMATOXYLIN AND EOSIN CELL STAINING PROCEDURE

- (1) Stain cells for 20-30 min in Erhlich's Haematoxylin (Sigma Diagnostics).
- (2) Place slide in a Coplan jar filled with an alkaline water solution until cells turn blue and are overstained.
- (3) Dip the slide in a Coplan jar filled with distilled water and 6 drops of concentrated hydrochloric acid for a few seconds until the cells turn red.
- (4) Turn cells back blue by placing the slide in an alkaline water solution.
- (5) Examine slide by light microscopy for cells with bright blue stained nuclei and unstained cytoplasm. If this is not the case, continue steps 2-4 until so.
- (6) Wash slide for 5 min under running water, pat dry with blotting paper.
- (7) Counterstain for 3 min with 0.1% aqueous Eosin Y (Sigma Diagnostics).
- (8) Wash in running water for 2 min.
- (9) Air dry, mount in glycerol with a coverslip.
- (10) Examine cells by light microscopy.