INVESTIGATION OF THE MECHANISM OF FENFLURAMINE-INDUCED PULMONARY PHOSPHOLIPIDOSIS IN THE RAT LUNG MODEL.

by

M.S. HASSAN

INVESTIGATION OF THE MECHANISM OF FENFLURAMINE-INDUCED PULMONARY PHOSPHOLIPIDOSIS IN THE RAT LUNG MODEL.

By

MOGAMAT SHAFICK HASSAN

B.Pharm (UWC)

Dissertation submitted in partial fulfilment of the requirements for the degree of M.Pharm. in the Department of Pharmacology, School of Pharmacy, University of the Western Cape.

Supervisor: Professor J.A. Syce
Chairperson of the Department of Pharmacology
University of the Western Cape
Bellvile, South Africa

March 1993

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude and appreciation to the following:

Prof J.A. Syce, my supervisor, who provided the impetus for this project. His inspiration, excellent supervision, insight and encouragement throughout this project will always be remembered.

Nazlia Samodien (Department of Pathology) and Pam Delport (Department of Anatomy), University of the Western Cape, for the preparation of the slides for light microscopy.

Basil Julies, Department of Physics, University of the Western Cape, for his expert technical asistance with the electron microscope.

Keryn Woolward, Department Biological Sciences, Peninsula Technikon, for the useful discussions and examination of the light microscopic slides.

Dr. Cridland, Department of Pharmacology, University of Cape Town, for allowing the use of the densitometer.

Lotter Lackay, Department of Analytical Sciences, Peninsula Technikon, for allowing the use of the differential scanning calorimeter (DSC).

Department of Chemistry, University of Cape Town, for the NMR analysis.

John Mathews, Department of Para-Medical Sciences, Peninsula Technikon, for his encouragement and valuable advice on the personal computer.

Magdalene Baumann, Peninsula Technikon, for her excellent co-operation in setting and aiding with the layout of this dissertation.

Bruno Pomario, Thys Mouton and Christo Bester, my colleagues at the Department of Para-Medical Sciences, Peninsula Technikon, with whom I shared a pleasant and helpful relationship. I am deeply indebted to Bruno for his advice and for reviewing this manuscript.

Mr Y. Alexander for his expert technical and photographic assistance.

Peninsula Technikon, for their generous financial support to facilitate the reproduction and binding of this dissertation.

My father, mother and sisters, for their support, encouragement and interest in my work which has been a continual source of inspiration.

All members of the staff of the School of Pharmacy, University of the Western Cape and Department of Para-Medical Sciences, Peninsula Technikon for their interest shown in my work.

DEDICATION

To my wife, Quanita and son Junaid, for their understanding, patience, support and constant encouragement.

CONTENTS PAGE

LIST OF TABLES ix

LIST OF FIGURES xii

CHAPTER 1 INTRODUCTION 2

CHAPTER 2 LITERATURE BACKGROUND 5

2.1 Pulmonary phospholipidosis 5

2.2 Drug-induced phospholipidosis 8

2.3 Mechanism of phospholipid accumulation 10

2.4 Chlorphentermine 14

2.4.1 Disposition in the lung 15

2.4.2 Phospholipids induced 16

2.5 Fenfluramine 17

2.5.1 Disposition in the body 17

2.5.2 Phospholipids induced 18

2.6 Models to study phospholipidosis 20

CHAPTER 3 PLAN OF WORK 23

3.1 Test model for drug-induced pulmonary phospholipidosis 24

3.2 The effect of fenfluramine on lung phospholipids . . . 25

3.3 The effect of fenfluramine on phospholipase activity . 26

3.4 Assesment of the extent of fenfluramine binding to the phospholipids 27

CHAPTER 4 MATERIALS AND METHODS 30

4.1 Materials, equipment and accessories 30

4.2 Methods 35

4.2.1 Preparation of the test drugs 35

4.2.1.1	Extraction of fenfluramine	35
4.2.1.2	Purification of fenfluramine	36
4.2.1.3	Synthesis of chlorphentermine	39
4.2.2	Induction of phospholipidosis	43
4.2.2.1	Drug administration and dosing	43
4.2.3	Analysis of rat lungs	43
4.2.3.1.	Procedure for unlavaged lungs	45
4.2.3.1.1	Histological studies	45
4.2.3.1.1.1	Light microscopy	45
4.2.3.1.1.1.2	Fixing of lung portions	46
4.2.3.1.1.1.3	Processing procedure	46
4.2.3.1.1.1.4	Special staining of lung sections	47
4.2.3.1.1.1.4.1	Reagents	47
4.2.3.1.1.1.4.2	Procedure for Haematoxylin and Eosin stain	49
4.2.3.1.1.1.4.3	Procedure for Sudan VI	51
4.2.3.1.1.2	Electron microscopy	52
4.2.3.1.1.2.1	Reagents	52
4.2.3.1.1.2.2	Procedures common to SEM and TEM	54
4.2.3.1.1.2.3	Procedure for SEM	55
4.2.3.1.1.2.4	Procedure for TEM	56
4.2.3.1.1.2.5	Procedure for double staining of ultrathin sections	57
4.2.3.2	Procedure for lavaged lungs	58
4.2.3.2.1	Isolation of alveolar macrophages	58
4.2.3.2.2	Hank's balanced salt solution	59
4.2.3.2.3	Enumeration of the macrophages	59
4.2.3.3	Procedures common to both unlavaged and lavaged lungs	60
4.2.3.3.1	Extraction of lipids	60
4.2.3.3.1.1	Extraction procedure	61
4.2.3.3.2	Chromatography	62
4.2.3.3.2.1	Solvent systems	62
4.2.3.3.2.2	Sample application and chromatographic separation	63

4.2.3.3.2.3	Densitometry	64
4.2.3.3.2.4	Quantitation of phospholipids	64
4.2.4	Assay for phospholipase activity	65
4.2.4.1	Reagents	65
4.2.4.2	Phospholipase-A activity	67
4.2.4.3	Preparation of solutions	67
4.2.4.4	Hydrolysis of phospholipid	69
4.2.4.4.1	Protocol	69
4.2.4.4.2	Procedure	69
4.2.4.5	Determination of the liberated fatty acids.	70
4.2.4.6	Quantitation of fatty acid concentration, PL-A activity and % inhibition of PL-A activity	71
4.2.4.7	Phospholipase C activity	73
4.2.4.7.1	Preparation of solutions	73
4.2.4.8	Hydrolysis of phospholipid	75
4.2.4.8.1	Protocol	75
4.2.4.8.2	Procedure	75
4.2.4.9	Determination of liberated inorganic phosphate	76
4.2.4.9.1.	Method	76
4.2.4.9.2	Calculation of inorganic phosphorus concentration	77
4.2.5	Differential scanning calorimetric determination of the phospholipid phase transition temperature	79
4.2.5.1	Reagents	80
4.2.5.2	Preparation of solutions	80
4.2.5.3	Determination of the phospholipid phase transition temperature	80
4.2.5.3.1	Protocol	80
4.2.5.3.2	Procedure	81
4.2.5.3.3	Phase transition temperature measurement	81
4.2.5.4	Analysis of the phase transition temperature	82

CHAPTER 5 **RESULTS AND DISCUSSION** 84

5.1 Body mass and food intake 84

5.2 Identification of foam cells in the alveolar sacs 88

5.3 Enumeration of alveolar macrophages 109

5.4 Phospholipid composition of unlavaged and lavaged lungs 111

5.5 Effects of chlorphentermine and fenfluramine on phospholipase activity 124

5.6 Effects of chlorphentermine and fenfluramine on the phase transition temperature of phospholipids 132

5.6.1 Correlation of the effects of chlorphentermine and fenfluramine on the phase transition temperature, phospholipase inhibition and lung phospholipid levels 139

CHAPTER 6 **CONCLUSION** 149

SUMMARY 154

LIST OF REFERENCES 158

CURRICULUM VITAE 174

LIST OF TABLES

PAGE

TABLE 1 Mass of Wistar rats after chronic administration of chlorphentermine, fenfluramine or saline. 85

TABLE 2 Mass of BD9 rats after chronic administration of chlorphentermine, fenfluramine or saline. 85

TABLE 3 Macrophage counts for lavaged lungs of Wistar rats after treatment with chlorphentermine, fenfluramine and saline 110

TABLE 4 Macrophage counts for lavaged lungs of BD9 rats after treatment with chlorphentermine, fenfluramine and saline 110

TABLE 5 Phospholipid levels (mg/g dry lung ± SD) in unlavaged lungs of Wistar rats treated with chlorphentermine, fenfluramine or saline 114

TABLE 6 Phospholipid levels (mg/g dry lung ± SD) in unlavaged lungs of BD9 rats treated with chlorphentermine, fenfluramine or saline 115

TABLE 7 Phospholipid levels (mg/g dry lung ± SD) in lavaged lungs of Wistar rats treated with chlorphentermine, fenfluramine or saline 116

TABLE 8 Phospholipid levels (mg/g dry lung ± SD) in lavaged lungs of BD9 rats treated with chlorphentermine, fenfluramine or saline 117

TABLE 9 PL-A activity following the enzymatic breakdown of phosphatidyl choline 121

TABLE 10 PL-A activity following the enzymatic breakdown of phosphatidyl ethanolamine 121

TABLE 11 PL-A activity following the enzymatic breakdown of phosphatidyl glycerol 122

TABLE 12 PL-A activity following the enzymatic breakdown of phosphatidyl inositol 122

TABLE 13 PL-A activity following the enzymatic breakdown of phosphatidyl serine 123

TABLE 14 PL-A activity following the enzymatic breakdown of sphingomyelin 123

TABLE 15 PL-A activity following the enzymatic breakdown of phosphatidic acid 124

TABLE 16 PL-C activity following the enzymatic breakdown of phosphatidyl choline 126

TABLE 17 PL-C activity following the enzymatic breakdown of phosphatidyl ethanolamine 126

TABLE 18 PL-C activity following the enzymatic breakdown of phosphatidyl glycerol 127

TABLE 19 PL-C activity following the enzymatic breakdown of phosphatidyl inositol 127

TABLE 20 PL-C activity following the enzymatic breakdown of phosphatidyl serine 128

TABLE 21 PL-C activity following the enzymatic breakdown of sphingomyelin 128

TABLE 22 PL-C activity following the enzymatic breakdown of phosphatidic acid 129

TABLE 23 Phase transition temperature of phosphatidyl choline 132

TABLE 24 Phase transition temperature of phosphatidyl ethanolamine . . 132

TABLE 25 Phase transition temperature of phosphatidyl glycerol 132

TABLE 26 Phase transition temperature of phoshatidyl inositol 133

TABLE 27 Phase transition temperature of phosphatidyl serine 133

TABLE 28 Phase transition temperature of sphingomyelin 133

TABLE 29 Phase transition temperature of phosphatidic acid 134

TABLE 30 Comparison of the phase transition temperature and percentage inhibition of PL-A and PL-C after complex formation between fenfluramine and various phospholipids. 143

TABLE 31 Comparison of the phase transition temperature and percentage inhibition of PL-A and PL-C after complex formation between chlorphentermine and various phospholipids. 143

LIST OF FIGURES

PAGE

FIGURE 1 Structure of cationic amphiphilic drugs 8

FIGURE 2 Cellular uptake and lysomal accumulation 9

FIGURE 3 Induction of specific phospholipids by cationic amphiphilic drugs 14

FIGURE 4 Structures of some anorectic drugs 19

FIGURE 5 Inducers of lipidosis in humans, animals and cultured cells . . 21

FIGURE 6 NMR spectrum ($CDCl_3$) of fenfluramine hydrochloride 37

FIGURE 7 IR spectrum (KBr) of fenfluramine hydrochloride 38

FIGURE 8 NMR spectrum ($CDCl_3$) of chlorphentermine hydrochloride 41

FIGURE 9 IR spectrum (KBr) of chlorphentermine hydrochloride 42

FIGURE 10 Flowchart of the analysis of treated and untreated rat lungs 44

FIGURE 11 Flow chart of the hydrolysis of phospholipids and the determination of PL-A activity 72

FIGURE 12 Flow chart of the hydrolysis of phospholipids and the determination of PL-C activity 78

FIGURE 13 Bar graph of the mean mass of Wistar rats after chronic administration of chlorphentermine, fenfluramine, or saline as a function of the duration of treatment 86

FIGURE 14 Bar graph of the mean mass of BD9 rats after chronic administration of chlorphentermine, fenfluramine, or saline as a function of the duration of treatment 87

FIGURE 15 Lung section of control rats (Wistar or BD9) treated with saline for 6 weeks. 90

FIGURE 16 Alveolar macrophages (AM) "foam cells" in the alveolar spaces (A) of Wistar rats treated with either fenfluramine (a) or chlorphentermine (b) at a dose of 40 mg/kg body weight for six weeks. 91

FIGURE 17 Alveolar macrophages (AM) "foam cells" in the alveolar spaces (A) of BD9 rats treated with either fenfluramine (a) or chlorphentermine (b) at a dose of 40 mg/kg body weight for six weeks. 93

FIGURE 18 No prominant evidence of phospholipid distribution in the alveolar epithelia (AE) and alveolar spaces (A) of the saline control rats (Wistar or BD9) after six weeks treatment. 93

FIGURE 19 Distribution of phospholipids (P) in the alveolar epithelia (AE), alveolar spaces (A) and alveolar macrophages (AM) of Wistar rats treated with fenfluramine or chlorphentermine at a dose of 40mg/kg body weight for six weeks. 94

FIGURE 20 Distribution of phospholipids (P) in the alveolar epithelia (AE), alveolar spaces (A) and alveolar macrophages (AM) of BD9 rats treated with fenfluramine or chlorphentermine at a dose of 40mg/kg body weight for six weeks. 95

FIGURE 21 Scanning electron micrograph of an alveolar macrophage (AM) in the alveolar spaces (A) of control rats (Wistar or BD9) treated with saline for six weeks 98

FIGURE 22 Scanning electron micrograph of alveolar macrophages "foam cells" in the alveolar spaces of Wistar rats treated with fenfluramine at a dose of 40 mg/kg body weight for six weeks. 99

FIGURE 23 Scanning electron micrograph of alveolar macrophages "foam cells" in the alveolar spaces of Wistar rats treated with chlorphentermine at a dose of 40 mg/kg body weight for six weeks.. 100

FIGURE 24 Scanning electron micrograph of alveolar macrophages "foam cells" in the alveolar spaces of BD9 rats treated with fenfluramine at a dose of 40 mg/kg body weight for six weeks. 101

FIGURE 25 Scanning electron micrograph of alveolar macrophages "foam cells" in the alveolar spaces of BD9 rats treated with chlorphentermine at a dose of 40 mg/kg body weight for six weeks. 102

FIGURE 26 Transmission electron micrograph of an alveolar macrophage of a control rat (Wistar or BD9) treated with saline. 103

FIGURE 27 Transmission electron micrograph of phospholipid rich lamellar bodies as it occurs in the alveolar macrophages. 104

FIGURE 28 Transmission electron micrograph of an alveolar macrophage "foam cell" in the alveolar space of a Wistar rat treated with fenfluramine at a dose of 40mg/kg body weight for six weeks. 105

FIGURE 29 Transmission electron micrograph of an alveolar macrophage "foam cell" in the alveolar spaces of Wistar rats treated with chlorphentermine at a dose of 40mg/kg body weight for six weeks. 106

FIGURE 30 Transmission electron micrograph of an alveolar macrophage "foam cell" in the alveolar spaces of BD9 rats treated with fenfluramine at a dose of 40mg/kg body weight for six weeks. 107

FIGURE 31 Transmission electron micrograph of an alveolar macrophage "foam cell" in the alveolar spaces of BD9 rats treated with chlorphentermine at a dose of 40mg/kg body weight for six weeks 108

FIGURE 32 Structures of phospholipids 135

CHAPTER ONE

INTRODUCTION

INTRODUCTION

Pulmonary phospholipidosis is a lipid storage disorder. It is characterised by the accumulation of lipo-proteinaceceous material in the alveolar epithelia and the excess accumulation of hypertrophied alveolar macrophages in the alveolar spaces. These hypertrophied alveolar macrophages are also commonly referred to as foam cells. Ultrastructural evidence indicates that the cytoplasm of such foam cells is characterised by concentric lamellar inclusion bodies. In turn histochemical and biochemical evidence indicate that the inclusion bodies are lysosomal in origin and predominantly phospholipid in nature[1,2]. The accumulated lipo-proteinaceous material is of interest to us because of its chemical similarity to normal pulmonary surfactant and the possible effects it could have on normal lung function.

Several cationic amphiphilic drugs have been shown to induce pulmonary phospholipidosis. Understanding how these drugs induce phospholipidosis may give us a better insight into pulmonary surfactant synthesis and how it may be altered. Furthermore the model of drug-induced phospholipidosis may prove useful in allowing us to investigate the functioning of pulmonary surfactant. Unfortunately little is known about the mechanism of this drug-induced phenomenon. Most of the work on the aetiology of drug-induced phospholipidosis has focused on the phospholipid involvement. Thus it is hypothesised that at least two possible mechanisms can account for the observed accumulation of phospholipids. Phospholipid accumulation may arise because of the inhibition of specific lysosomal phospholipases which, in turn, may occur due to direct binding of the drug to the phospholipases or indirectly by the drug raising the

intralysosomal pH[3,4]. Secondly, by increased binding of the drug to the phospholipids, the drug-phospholipid complex thus formed may inhibit phospholipase activity[5]. Significantly it has also been found that differences exist in the levels of phospholipids induced by different drugs[6]. Such differences may arise due to differences in the mechanisms by which these drugs cause phospholipids to accumulate. If this is correct, a detailed analysis of the levels and type of phospholipids induced by carefully selected drugs may give us a clearer insight into the mechanism of this drug-induced phenomenon.

One class of drugs implicated in this phenomenon is the anorexigenics eg. chlorphentermine and fenfluramine. Fenfluramine is still readily available on the market but how it may produce phospholipidosis has not been studied. As a full scale clinical study into the aetiology of the drug-induced phenomenon of pulmonary phospholipidosis at this stage may not be justified, it was decided to use a rat model to compare the levels of accumulated phospholipids which arise after treatment with these two anorexigenic drugs. The strategy adopted was to use a closely related drug viz. chlorphentermine, which is well known to induce phospholipidosis, to investigate the mechanism for fenfluramine-induced phospholipidosis. In doing so, we believe the discerning power of the experiment was made as sensitive as possible. The hypothesis for this study was that fenfluramine would induce the elevation of the same phospholipids as chlorphentermine, because like chlorphentermine, it also induces phospholipidosis by either directly inhibiting lysosomal phospholipases and/or binding to the phospholipid substrate. To test the validity of this hypothesis, it was necessary to compare phospholipid levels as well as investigate the mechanism of phospholipase inactivation that could be involved.

CHAPTER TWO

LITERATURE BACKGROUND

LITERATURE BACKGROUND

2.1 PULMONARY PHOSPHOLIPIDOSIS

Phospholipidosis is a phospholipid storage disorder occuring in many tissues of the body with the lung being severely affected[3]. Lung biopsies reveal diffuse pathological changes with characteristic cytoplasmic inclusions in the lung parenchyma cells, including endothelial, alveolar epithelial and interstitial cells as well as the alveolar macrophages[7]. The disorder is characterised by a profound accumulation of hypertrophied alveolar macrophages[3]. Because of their general microscopic appearance these hypertrophied alveolar macrophages are also commonly referred to as "foam" cells.

Ultrastructural evidence indicates that the cytoplasm of such foam cells are characterised by concentric lamellar inclusion bodies. These inclusion bodies which have a periodicity of 4-5 nm appear to disintegrate, with time, into membranous and granular debris[2]. Because the lamellar inclusions contain acid phosphatase and β-glucuronidase activities it is believed that they are lysosomal in origin[1]. In addition, the cells are phagocytic[8], and their ingested material is associated with the lamellar inclusions. Further the ultrastructure of the inclusion bodies closely resembles that of phospholipid micelles in the water phase[2].

The accumulated lipo-proteinaceous material contained in the inclusion bodies closely resembles normal pulmonary surfactant in composition.

Isolated surfactant consists of 90 % phospholipid with neutral lipids and proteins making up the rest. The neutral lipids consists of di- and triglycerides and cholesterol, with cholesterol representing 40-60 % of this fraction[9,10,11]. Surfactant contains the following phospholipid components: phosphatidyl choline 51-92 %, phosphatidyl glycerol ca[a]. 5 %, phosphatidyl ethanolamine ca. 6 %, phosphatidyl inositol and phosphatidyl serine together ca. 5 % and sphingomyelin ca. 2 %[12]. Wide fluctuation in values for the levels of such phospholipids appear in literature, probably due to differences in experimental techniques used by the different investigators. It may however also reflect species differences. The phospholipid, phosphatidyl choline contains two saturated acyl chains, and the DPPC[b] form predominates in surfactant[12]. This phospholipid is the major surface-active component of pulmonary surfactant that is responsible for decreasing surface tension in the lung[13]. The lipo-proteinaceous material also contains a number of proteins, including serum albumin. Four families of lung specific apoprotein have been described viz. SP[c]-A, SP-B, SP-C and SP-D with molecular masses of 35 000, 10 000, 10 000 and 43 000 dalton, respectively[14,15].

Lung surfactant is present in two major pools: extracellular and intracellular pools. Most of the analytical data has been obtained on extracellular surfactant which are secreted into the alveoli and which can be isolated by lung lavage[12]. Intracellular lung surfactant, present as

---

a ca. = approximately

b DPPC = L-α-dipalmitoyl phosphatidyl choline

c SP = surfactant-associated protein

lamellar bodies in the alveolar type II cells, has not been studied as extensively as the extracellular material. When it has been isolated, the intracellular surfactant however showed a similar composition to that of the isolated extracellular material[12].

Evidence indicates that as the alveolar macrophages become larger, their total phospholipid content increases proportionally[4]. In diseases characterised by phospholipidosis, surfactant and macrophage levels are elevated simultaneously[12]. It was found that increasing amounts of alveolar surfactant preceded the influx of macrophages in the alveoli[16]. This suggested that surfactant phospholipids could induce a chemotactic movement of macrophages[17].

Phospholipidosis have been extensively described, and may occur as an inherited disease. Genetically determined specific enzyme deficiencies are the cause of several lysosomal lipid storage disorders[2]. In all cases the defective or missing enzyme is one specifically required for the catabolism of a lipid which accumulates in the tissues of the affected individual. Examples of these diseases include Niemann-Pick disease, characterised by the accumulation of sphingomyelin in the brain, liver, and spleen[18] and Tay Sachs disease which is characterised by the accumulation of ganglioside G_{M2} in the brain[19]. Phospholipidosis is however also a drug-induced condition caused by drugs that vary widely in their principle pharmacological actions[2].

2.2 DRUG-INDUCED PHOSPHOLIPIDOSIS

More than 30 xenobiotics, including chloroquine, iprindole, imipramine and chlorpromazine, have been identified to cause lipid storage disorders in humans, animals or cultured cells[20]. Frequently, but not always, doses far exceeding the effective pharmacological dose are required to produce the lipid accumulation[6]. A common feature of these drugs is their cationic amphiphilic nature. The cationic-amphiphilic features of these drugs include the presence of (a) one or several aromatic rings, the hydrophobic nature of which may be enhanced by an appropriate substitute e.g. halogen and, (b) a side chain, with an amine group which is highly hydrophilic and predominantly ionised at the physiological pH (fig 1).

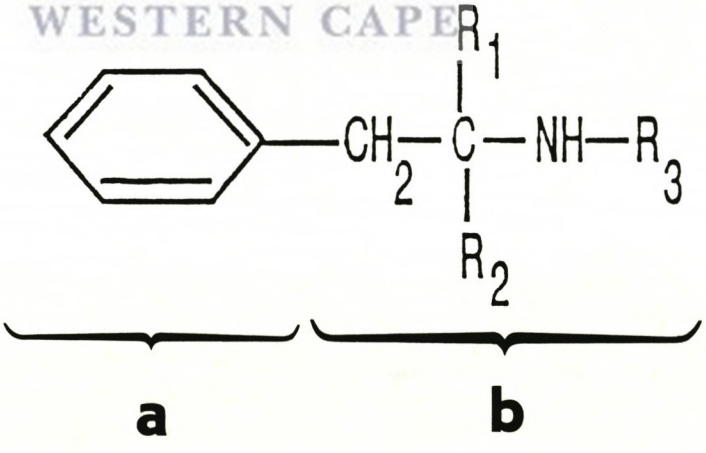


Fig 1. Structure of cationic amphiphilic drugs[d].

d R_1, R_2 and R_3 = side chains

For a drug to induce pulmonary phospholipidosis, it must penetrate the plasmalemma of the target cell readily and concentrate in the lysosomes[3]. The penetration rate will depend on (a) the pKa of the amine group and (b) the degree of hydrophobicity of the entire molecule. If the apolarity is extremely pronounced, even a protonised amine group will not completely hinder the penetration of the molecule through the plasmalemma[5]. The unprotonated form of the drug gains access readily into the lysosomes by diffusion. Due to the low pH in the lysosomes, the drug is rapidly protonated and thus trapped in the acidic environment. The protonated cationic amphiphilic drug binds to the phospholipids, and the drug-phospholipid interaction creates a concentration gradient for more diffusion of drug into the lysosome. Accumulation of the drug in the lysosomes proceeds as long as the pH there remains low (fig 2). The exact mechanism for the accumulation of the phospholipid is however less clear.

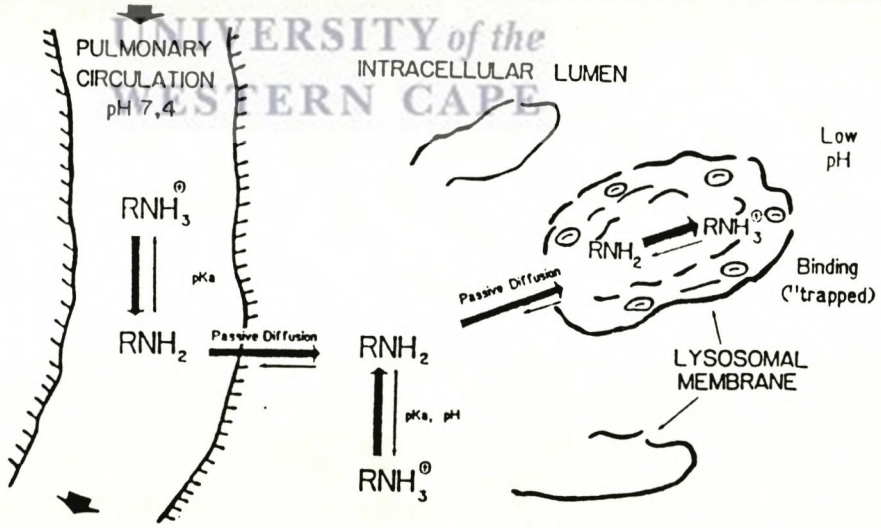


Fig.2 Cellular uptake and lysosomal accumulation[e,f]

e RNH_2 = non-ionised cationic amphiphilic drug

f NH_3^+ = ionised cationic amphiphilic drug

2.3 MECHANISM OF PHOSPHOLIPID ACCUMULATION

Indeed, it is hypothesised that various mechanisms could account for the observed accumulation of phospholipids in the alveolar macrophages.

(a) Inhibition of specific lysosomal phospholipases.

This may occur directly by complexation of the drug to the phospholipases or indirectly due to the accumulation of the unbound protonated drug in the lysosomes raising the pH. The raised pH could significantly depress phospholipase-A and -C activity[3,4].

(b) Complex formation between drug and phospholipid.

The drug-phospholipid complex results in an accumulation of phospholipids within the lysosomes. Firstly, due to the modification of the substrate the drug-phospholipid complex inhibits or is resistant to phospholipase activity. Secondly, a gradient for polar phospholipids is sustained from the cytosol into the lysosomes, since the drug-phospholipid complex is removed from the phospholipid diffusion equilibria[5].

(c) Increased phospholipid synthesis which exceeds the degrading capacity of the phospholipases.

Much work has been done on mechanism (b) and it is postulated that the interaction between the phospholipid and drug may be due to an electrostatic attraction between the negatively charged phosphate moiety of the phospholipid and the positively charged nitrogen atom of the drugs[2].

This possibility is strengthened by the fact that no compound with an anionic side chain has been reported to induce phospholipidosis. In the second phase of the interaction hydrophobic bonds might cause an association of the aromatic ring to the hydrophobic moiety of the phospholipid molecule (or the phospholipid micelle). The tendency to form an association of this kind can be expected to be enhanced with increasing apolarity of the ring system[2].

It is apparent from the brief discussion above that the etiology of drug-induced phospholipidosis may well be very complex with several mechanisms being involved. A number of investigators have to date illustrated that drug-induced phospholipidosis may be caused by (a) inhibition of specific lysosomal phospholipases[21], or (b) an increase binding of the drug to the phospholipid[2]. These findings make a compensatory stimulation of phospholipid synthesis as a consequence of a functional deficiency unlikely[2].

If drug-induced phospholipidosis is the result of inhibition by the drug of a phospholipid-degrading enzyme, it should be possible to demonstrate both the specific lipid accumulation and the associated enzyme deficiency in the affected tissue[22]. Animals treated with AY-9944 (trans-1,4-bis[2-chlorobenzylaminomethyl] cyclohexane dihydrochloride) presents with lamellar inclusion bodies in the retina and liver[6]. These morphological changes are associated with the accumulation of sphingomyelins in the liver, as well as a 50 to 90 % reduction of sphingomyelinase activities in various tissues. Concentrations of other liver lipids are essentially

unchanged in the rat after treatment with AY-9944[23].

Other examples exist of marked accumulation of individual lipids in association with drug-induced lysosomal inclusions, but the corresponding enzyme deficiency has not been demonstrated (see figure 3). The most striking of these occur in the liver of rats receiving the coronary vasodilator DH (4,4'bis-(di-ethylaminoethoxy)). Analysis of liver tissue showed marked increases in the content of the acidic phospholipids BMGP (bis-(monoacylglyceryl) phosphate)[24], phosphatidyl inositol[25], phosphatidyl glycerol[24] and sphingomyelin[26]. Lesser elevation of phosphatidyl choline and phosphatidyl ethanolamine was also observed. In a study done on the rat kidney after treatment with gentamicin and chlorphentermine, the latter produced significant rises in renal phosphatidyl inositol, phosphatidyl serine, phosphatidyl ethanolamine, phosphatidyl glycerol and phosphatidyl choline. Gentamicin in turn produced significant rises in the phosphatidyl inositol, phosphatidyl serine, and phosphatidyl choline but no marked alterations in sphingomyelin, phosphatidyl ethanolamine and phosphatidyl glycerol levels[27]. It appears that the induction of phospholipidosis by different drugs may result in the accumulation of different lipid classes, indicating that the effects of these drugs are not always the same. If this is correct the question may be asked, why different drugs induce the accumulation of different phospholipids.

A number of cationic amphiphilic drugs show an increased binding to the phospholipids in the following order: sphingomyelin = phosphatidyl

choline < phosphatidyl ethanolamine < phosphatidyl serine[28]. It has been proposed that hydrophobic and hydrophilic binding of drugs to phospholipids is the most common cause of drug-induced pulmonary phospholipidosis[5]. Several drugs of varying phospholipidotic capacity were tested in vitro for their tendency to bind at the hydrophobic and hydrophilic sites on phosphatidyl choline. Phosphatidyl choline was used since it is the major component of lung surfactant and cationic amphiphilic drugs are known to increase phosphatidyl choline levels to a greater extent when compared to other phospholipid classes in the lung[29]. Amiodarone was found to bind strongly to the hydrophobic part and chlorphentermine strongly to the hydrophilic part of phosphatidyl choline, whereas trimipramine binds with equal strengths to both the hydrophobic and hydrophilic parts of the phosphatidyl choline molecule. Chloroquine on the other hand did not bind to either the hydrophobic or hydrophilic part of phosphatidyl choline. Chloroquine has however been reported to have a high affinity for negatively charged phospholipids like phosphatidyl serine[30]. It is likely that the presence of two amino groups on the polar side chain of chloroquine creates electrostatic repulsions, from the positively charged PC molecules. Literature reports indicate that chloroquine-induced phospholipidosis occurs as a result of inhibition of lysosomal phospholipases[31]. Different drugs may thus induce the elevated levels of different phospholipids because of differences in their mechanisms of phospholipidosis induction.

Drug	Species	Tissue	Phospholipid
Triparanol	Hamster	Adrenal	PS,PI,PA[6]
DH[g]	Rat	Liver	BMPG[h],PI,PS[26]
Chloroquine	Rat	Liver	BMPG,PI,PS[32]
Chlorphentermine	Rat	Lung	PC,SPM,PE,PS,PI,PG[33]
1-Chloramitryptiline	Rat	Lung	PA[34]
RMI 10,393	Rat	Lung	PC,PA[34]
Amiodarone	Human	Lung lavage fluid	PC,PG,PE,BMPG,PS,PI[35]
Chlorocyclizine	Rat	Lung	PC,PG[36]
Gentamicin	Rat	Kidney	PI,PS,PC[27]

Fig 3. Induction of specific phospholipids by cationic amphipilic drugs

2.4 CHLORPHENTERMINE

Chlorphentermine (o-chloro-α-dimethylphenethylamine) is an appetite suppressant which was marketed in South Africa under the trade name Pre-Sate®. It is a sympathomimetic agent which is claimed to have little stimulant effect on the central nervous system. Several years after being on the market, it was found to increase the pulmonary arterial blood pressure and alter the morphology of rat lungs after chronic administration. Mainly as a result of this, chlorphentermine was withdrawn from the market in many countries[37].

g DH = 4,4'bis-(di-ethylaminoethoxy)

h BMGP = bis-(monoacylglyceryl) phosphate, PC = phosphatidyl choline, PE = phosphatidyl ethanolamine, PS = phosphatidyl serine, PI = phosphatidyl inositol, PG = phosphatidyl glycerol, SPM = spingomyelin, PA = phosphatidic acid.

2.4.1 DISPOSITION IN THE LUNG

Chlorphentermine is known to be exceptionally pneumophilic and accumulates preferentially in the lung relative to other tissues[38]. It accumulates in the lung by a sodium dependent uptake process coupled with a diffusion and binding process. It complexes with phospholipids and the chlorphentermine becomes rapidly protonated upon entering the lysosomes and this forms a slowly effluxable pool. This results in the avid accumulation and persistence of chlorphentermine in the lung[39].

Conjugation of chlorphentermine to a polar metabolite is the major route of metabolism in the rat. The polar metabolite so formed can be converted to free chlorphentermine by hydrolysis. Chlorphentermine is also metabolised more slowly than its unchlorinated analog, phentermine[40] (see fig 4, page 19). In contrast to chlorphentermine, phentermine does not display comparable pharmacokinetics and does not accumulate in the lung to any appreciable extent. The marked difference in the lung accumulation between these closely related compounds can only be due to the para-chlorine substitution on the benzene ring, which increases the amphiphilic nature of chlorphentermine. The difference in the pneumophilicity between these compounds is most probably due to the difference in affinity of these drugs for the binding sites in the lung. Thus the structural requirements for the accumulation of chlorphentermine appears to be quite specific. This difference in pneumophilicity may be the reason why pulmonary adverse effects are anticipated with chlorphentermine and not so much with phentermine.

Chlorphentermine also has the ability to induce the accumulation of pulmonary phospholipids[1].

2.4.2 PHOSPHOLIPIDS INDUCED

Within pulmonary tissue, the ability of chlorphentermine to induce an accumulation of phospholipids principally in the alveolar macrophages and unlavaged lung is well documented[3]. This makes chlorphentermine a useful drug for the study of drug-induced pulmonary phospholipidosis. The extent of the cellular accumulation of phospholipids after chlorphentermine administration is dependent on the duration of drug treatment and the dose at which it is administered[3,41,42]. Chronic, daily administration of approximately 50 mg/kg chlorphentermine for 4 to 8 weeks significantly elevates the concentration of PC[i], SPM[j] and PE[k] in the unlavaged lung[43]. With an increase in chlorphentermine dose to 80 mg/kg, a significant rise in rat lung PI[l], PC, and PG[m] is noted after two weeks[34]. At a dose of 30 mg/kg, chlorphentermine consistently induces a rise in the PC, PS[n] and PI levels after 4 weeks but at this dose no marked changes were reported in SPM and PE content of the lung[34].

---

i PC = phosphatidyl choline

j SPM = spingomyelin

k PE = phosphatidyl ethanolamine

l PI = phosphatidyl inositol

m PG = phosphatidyl glycerol

n PS = phosphatidyl serine

2.5 FENFLURAMINE

Fenfluramine (N-ethyl-α-methyl-3-trifluoromethyl-phenethylamine) is a widely used appetite suppressant. It is structurally related to amphetamine but is devoid of the many stimulant side effects such as anxiety, tremor and excitation characteristic of amphetamine. The lack of central nervous stimulation has been regarded as the major advantage of fenfluramine over amphetamine and fenfluramine also lacks the drug abuse potential associated with the latter[44].

Fenfluramine shares common therapeutic actions and chemical similarities with chlorphentermine. However it displays one structural difference which might account, in part, for the differences in its potency to induce lipidosis-like cellular alterations. The hydrophobic moiety of fenfluramine, contains a trifluoromethyl group in the m-position, which may be less apolar than the corresponding moiety of chlorphentermine, namely a chlorine atom located in ρ-position (figure 4). Thus the amphiphilic character of the fenfluramine molecule will be less pronounced than that of chlorphentermine[45]. In addition, differences in distribution and metabolism may also play a role[39].

2.5.1 DISPOSITION IN THE BODY

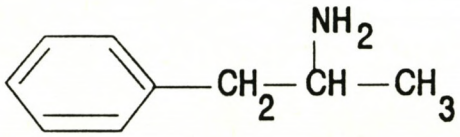
Fenfluramine is widely distributed to almost all tissues and it is rapidly converted to its major metabolite norfenfluramine[46]. In dogs treated with intravenous or oral doses of 5 mg/kg the percentages of total

fenfluramine and norfenfluramine in various organs 12 hours after the initial dose were 10 % in the liver, 3.5 % in the brain and 2.8 % in the lungs. Studies show that fenfluramine is also extensively metabolised in the rat lung and liver. Fenfluramine is less pneumophilic than chlorphentermine, thus the chance of inducing adverse effects in the lung is greatly reduced[46]. After oral administration, fenfluramine is subject to extensive first pass metabolism and about 60 % of the dose is metabolised to norfenfluramine. This metabolite may be detectable in plasma about 3 hours after ingestion. There is wide inter-individual variation in the rates of biotransformation and elimination of fenfluramine. Fenfluramine has also been shown to be metabolised to other compounds which, unlike norfenfluramine, are pharmacologically inactive. After initial and extensive dealkylation of fenfluramine to norfenfluramine, further metabolic transformation to 3-trifluoromethylbenzoic acid and subsequent excretion as its glycine conjugate, 3-trifluoromethylhippuric acid takes place[44]. Fenfluramine also has the ability to induce the accumulation of pulmonary phospholipids.

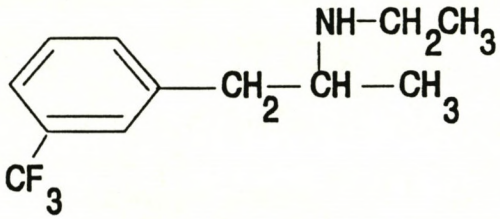
2.5.2 PHOSPHOLIPIDS INDUCED

Fenfluramine was found to cause milder cellular alterations, when compared with chlorphentermine[45]. After an extensive literature search no data was found to indicate which phospholipids may accumulate in the lung after treatment with fenfluramine. Considering the fact that there are differences in the phospholipid levels induced by the different cationic amphiphilic drugs and that such differences may arise due to differences

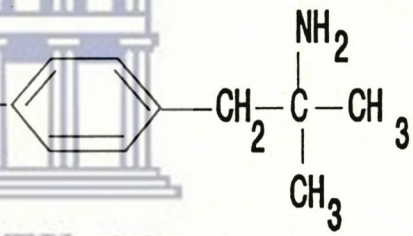
AMPHETAMINE



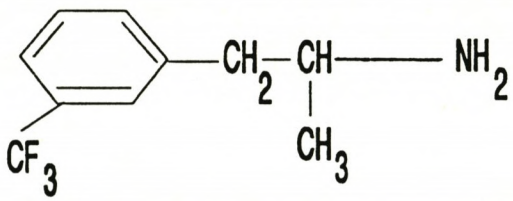
FENFLURAMINE



CHLORPHENTERMINE



NORFENFLURAMINE



PHENTERMINE

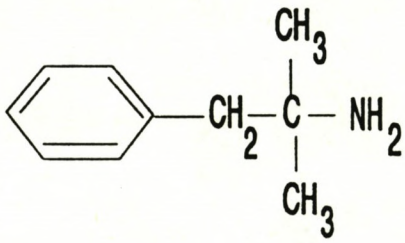


Figure 4. Structures of some Anorectic Drugs

in the mechanisms by which these drugs cause phospholipids to accumulate, a major objective of this thesis was to analyse the levels and type of phospholipids induced by fenfluramine. Such information may give us a clearer insight into the mechanism of this drug-induced phenomenon.

2.6 MODELS TO STUDY PHOSPHOLIPIDOSIS

Accumulation of phospholipids has been biochemically shown to occur in animals, cultured cells and humans[7](fig 5). The induction of this disorder in alveolar macrophages by chlorphentermine is species dependent. The rat and guinea pig are very susceptible while the mouse is less so and phospholipidosis is induced in the rabbit only after long periods of treatment, if at all[45]. While the basis for the differences in susceptibility has not been examined, it probably relates to differences in drug distribution and/or metabolism among these species[3]. We used two strains of rats in this investigation viz. Wistar and BD9, to account for the possibility of the drugs being more or less effective at inducing phospholipidosis because of the strain of rat used.

(1) INDUCERS OF LIPIDOSIS IN ANIMALS AND HUMANS

DRUG	CLASSIFICATION
Chloroquine	antimalarial[47]
Amiodarone	anti-arrythmic[35],[48]
Perhexiline	anti-anginal[5]

(2) INDUCERS OF LIPIDOSIS IN ANIMALS

DRUG	CLASSIFICATION
Imipramine	anti-depressant[49]
Clomipramine	anti-depressant[49]
Chlorphentermine	anorectic[38]
Fenfluramine	anorectic[45]
Triparanol	hypocholesterolemic[50]
AY-9944	hypocholesterolemic[50]
Chlorcyclizine	anti-histamine[5]

(3) INDUCERS OF LIPIDOSIS IN CULTURED CELLS WITH LOW LIPIDOSIS-INDUCING EFFICACY IN WHOLE ANIMALS

DRUG	CLASSIFICATION
Chlopromazine	neuroleptic[49]
Amitryptyline	antidepressant[49]
Lysergic acid	psychtropic drug[5]

Fig. 5 Inducers of lipidosis in humans, animals and cultured cells.

CHAPTER THREE

PLAN OF WORK

PLAN OF WORK

The objective of this study was to determine the pulmonary phospholipid levels as well as investigate the mechanism of phospholipase inactivation induced by fenfluramine, in the rat. This objective was firmly based on the hypothesis that fenfluramine like chlorphentermine would induce the elevation of the same phospholipids, because they each induce phospholipidosis by the same mechanism i.e. either by directly inhibiting lysosomal phospholipases and/or binding to the phospholipids. To realise the objective it was necessary to do the following:

(i) induce phospholipidosis in a suitable test model,

(ii) determine the effect of fenfluramine on lung phospholipids levels,

(iii) determine whether fenfluramine inhibits phospholipid degradation by direct inhibition of phospholipases or by phospholipid-substrate modification, and

(iv) assess the extent of fenfluramine binding to the phospholipids.

The general approach adopted in this study was to compare the effects of fenfluramine to that of chlorphentermine. The latter served as a good control as it is known to be exceptionally pneumophilic[38], and induces the formation of foam cells in the alveolar spaces[1]. After chronic administration of fenfluramine, chlorphentermine or saline to the rats, the tissue samples obtained from these rats were used in all the experiments set out below.

3.1 TEST MODEL FOR DRUG-INDUCED PULMONARY PHOSPHOLIPIDOSIS

To assess the effect of fenfluramine we needed a suitable model. The rat model was chosen for this study since rats were readily available and the condition under investigation could be induced in them with relative ease[3]. We used both BD9 and Wistar rats to ascertain whether this phenomenon was also strain dependent. As evidence for pulmonary phospholipidosis we looked for the presence of foam cells in the alveoli spaces. Consequently both light and electron microscopic studies were included in this investigation. In the light microscopic studies the following stains were used:

(a) The haemotoxylin and eosin stain to confirm the presence of foam cells in the alveolar spaces and their distribution throughout the lung tissue and,

(b) The Sudan IV stain was used to confirm the increase in total phospholipid levels in the lungs, especially in the alveolar macrophages, of the fenfluramine-and chlorphentermine-treated animals compared to the saline-treated controls.

The electron microscopic studies permitted the observation of cell and tissue structures beyond that seen with the light microscope and use was made of two types of electron microscopic techniques, viz.

(i) scanning electron microscopy (SEM), in which the surface morphology of the alveolar macrophages could be observed, and

(ii) transmission electron microscopy (TEM), in which a cross section of the alveolar macrophages could be observed and evidence for the presence of lamellar bodies could be found.

Phospholipidosis is characterised by elevated numbers of hyperthrophied alveolar macrophages in the alveolar spaces[3]. To determine the influence of fenfluramine on alveolar macrophage levels in the lung, a group of lungs from fenfluramine-, chlorphentermine-, and saline-treated rats were consequently also lavaged and the alveolar macrophages in the lavaged material enumerated by means of a Coulter® counter.

3.2 THE EFFECT OF FENFLURAMINE ON LUNG PHOSPHOLIPIDS

The idea was that by quantitating the individual phospholipid levels induced by fenfluramine and identifying those phospholipids showing unusual increases in their concentrations, a clearer insight into the mechanism of fenfluramine-induced phospholipidosis could be found. Therefore the lipids were extracted from the freeze-dried lung tissue of animals treated with fenfluramine, chlorphentermine or saline. The lipids were extracted according to the method of Radin, which uses hexane-isopropanol (HIP)[51]. The major advantage of extracting with HIP instead of the chloroform and methanol used in the method of Folch[52], is that almost no protein and little non-lipids are extracted from the lung.

The extracted phospholipids were separated by one dimensional, thin-layer chromatography (TLC), using 'high performance' silica gel precoated plates. With such plates, both the resolution and speed of separation are improved. The one-dimensional separation further had two major advantages over two-dimensional separation, viz. separation could be obtained in a shorter period of time and more than one sample, including standards, could be applied to allow for positive identification.

3.3 THE EFFECT OF FENFLURAMINE ON PHOSPHOLIPASE ACTIVITY

To ascertain whether the increases in phospholipid levels induced by fenfluramine and chlorphentermine were due to phospholipase-A or phospholipase-C inhibition, we monitored the effect of these drugs on phospholipase-A and phospholipase-C mediated hydrolyses of selected phospholipids. Phospholipase-A consists of a mixture of phospholipase-A_1 and phopholipase-A_2. Phospholipase-A_1 hydrolyses the fatty acid from the 1 position and phospholipase-A_2 from the 2 position on the phospholipid[19]. The process could be monitored by following the fatty acid production. Phospholipase-C hydrolyses the phospholipid into a 1,2 diacylglycerol and a phosphobase[19]. The formed phosphobase is then hydrolysed by alkaline phosphatase to liberate the inorganic phosphate. The inorganic phosphate is then detected colorimetrically. In order to minimize interference and complication of the procedures, samples of pure phospholipases (instead of lung tissue preparations) were used in this study. The phospholipids used in this study included phosphatidyl

choline, phosphatidyl ethanolamine, phosphatidyl serine, phosphatidyl glycerol, phosphatidyl inositol, phosphatidic acid and sphingomyelin. Since we wanted to assess whether these drugs would inhibit phospholipases directly or act by forming a complex with the phospholipid, and in this way prevent the latter from being metabolised by the phospholipase, the following protocol was used:

(i) Phospholipase-A or -C activity was determined in the presence of a particular phospholipid only,

(ii) The phospholipid was shaken with fenfluramine or chlorphentermine allowing for complex formation before phospholipase-A or -C was added, or

(iii) Fenfluramine or chlorphentermine was shaken with phospholipase-A or -C to allow for direct interaction between fenfluramine or chlorphentermine and the phospholipase, before addition of the phospholipid.

3.4 ASSESSMENT OF THE EXTENT OF FENFLURAMINE BINDING TO THE PHOSPHOLIPIDS.

To ascertain whether the mechanism for the increases in phospholipid levels induced by fenfluramine and chlorphentermine favours the one involving the complex formation between the drug and phospholipid, we monitored the effect of these drugs on the phase transition temperature

of phospholipids. It is reported that the amount of depression of the phase transition temperature, is dependent on the degree of binding of the drug to the phospholipid. This inturn is governed by the geometry of the drug molecules, which determines its orientation and position on the phospholipid molecule. The phospholipids used in this study included phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl serine, phosphatidyl glycerol, phosphatidyl inositol, phosphatidic acid and sphingomyelin. Since we wanted to confirm whether these drugs complex with the individual phospholipids, and in this way prevent the latter from being metabolised by the phospholipases, the following protocol was used:

(i) The phase transition temperature of each particular phospholipid was determined individually and,

(ii) The phospholipid was shaken with fenfluramine or chlorphentermine allowing for complex formation and then the phase transition temperature was determined.

CHAPTER FOUR

MATERIALS AND METHODS

MATERIALS AND METHODS

4.1 MATERIALS, EQUIPMENT AND ACCESSORIES

The following were the drugs, chemicals, reagents, equipment and accessories used in the extraction of fenfluramine, the synthesis of chlorphentermine, the histopathological studies, the separation and quantitation of the phospholipids and the phospholipase activity studies.

(a) DRUGS

Fenfluramine hydrochloride

Extracted from Ponderax® tablets (Servier Laboratories) by the
Department of Pharmacology
University of the Western Cape
Bellville, 7530.

Chlorphentermine hydrochloride

Synthesized in the Department of Pharmacology
University of the Western Cape
Bellville, 7530.

(b) CHEMICALS, REAGENTS AND GASSES

All the chemicals used in this study including those not listed in this section, were of analytical grade purity.

ρ-α-dichlorotoluene

Riedel - de Haen
Aktiengesellschaft, Verkauf LC
Wunstorfer Strabe 40, D-3016.

Haemotoxylin, eosin, sudan IV, glutaraldehyde fixative, osmium tetroxide and lead nitrate all from,

Merck

Damstadt, Germany.

Silica Gel TLC Plates 60, 20x20 cm, 0.25 mm thick

Merck

Damstadt, Germany.

Phosphatidyl choline, phosphatidyl serine, phosphatidyl inositol, phosphatidyl glycerol, sphingomyelin and phosphatidic acid all from,

Sigma Chemical Company

P.O. Box 14508

ST. Louis, MO,

U.S.A. 63178-9916

Phospholipase-A and Phospholipase-C kits

Boehringer Mannheim GmbH

Biochemica

Sandhofer Str. 116

6800 Mannheim 31

Germany.

ERL 4206 (Vinyl cyclohexane dioxide), DER 736

(Diglycidyl ether of propylene glycol), NSA (Nonenyl succinic anhydride), S-1 catalyst

(Dimethyl aminoethanol) and Beem® capsules all from,

Wirsam (Pty) Ltd.

Durban

Republic of South Africa.

Butylated hydroxy toluene (BHT)

Fluka AG

CH-9470 Buchs,

Switzerland

Nitrogen gas

Afrox (Pty) Ltd.

Cape Town

South Africa.

(c) EQUIPMENT

Shimadzu CS 9000 densitometer

Shimadzu

Japan.

NMR Spectrometer, Model EM 360

Varian Instrument Division

Palo Alto,

California

94303

UV/VIS Spectrophotometer, Model DU40

Beckman Instruments, INC.

Palo Alto,

California,

94304

Centrifuge, Model TJ-6

Beckman Instruments, INC

Palo Alto,

California, 94304

Coulter Counter

Coulter electronics, INC.

Hialeah,

Florida,

U.S.A.

Freeze dryer, LSL Secfroid SR

Labotec

Cape Town

South Africa

Histokinette automatic tissue processor-Model E7606

British American Optical Company Ltd.

820 Yeovil Road

Slougen - Bucks - England

Universal sliding microtome

Perchert (Pty) Ltd.

Austria

Tissue embedding centre - Tissue Tek 11

Tech Division

Miles Laboratories,

Naperville, IL 60540

Sorvall MT 5000 ultramicrotome

Sorvall

Switzerland.

Hitachi X650 scanning electron microscope and

Hitachi H800 transmission electron microscope

Hitachi, Japan

Techne S3 Sample Concentrator

Laboratory and Scientific Equipment (LASEC)

Cape Town

South Africa

Mettler AE163 analytical balance

Mettler Instruments

CH-8606

Greifensee

Zurich

Switzerland.

Du Pont 910 Differential Scanning Calorimeter (DSC)

Premier Technology

P O Box 173

Northriding

Johannesburg

2194, RSA

(d) RATS

BD9 rats

Medical Research Council

Tygerberg

Parow Valley

South Africa.

Wistar rats

Provincial Animal Centre

Private bag X06

Kuils River

South Africa.

(e) RAT FOOD

Epol (Pty) Ltd.
P.O. Box 3006
Johannesburg
South Africa

4.2 METHODS

4.2.1 PREPARATION OF THE TEST DRUGS

The fenfluramine and chlorphentermine used in this study were extracted, synthesized and purified as previously performed in our laboratory[53].

4.2.1.1 EXTRACTION OF FENFLURAMINE

Ninety Ponderax® tablets were crushed, and then mixed with 1 litre of 1N HCl, stirred for 24 hours and the resulting mixture allowed to settle overnight at room temperature (ca. 22° C). The aqueous phase was then decanted into large centrifuge tubes and centrifuged at 1250 g for 15 minutes. The supernatants were combined and filtered. The resulting solution was alkalinized with sodium hydroxide pellets and extracted with diethyl ether. The combined ether extracts were subsequently evaporated to a small volume (ca. 100 ml) on a rotary evaporator and dried over anhydrous sodium sulphate, filtered and stored in the cold room at 4° C until required for salt formation. To form the hydrochloride salt, hydrochloric acid gas was bubbled through the ethereal solution which turned cloudy as the salt was formed. The ether was then evaporated off,

the remaining residue frozen in a liquid nitrogen bath and freeze-dried. Before purification the dry crystals were washed with sodium-dried diethyl ether, filtered and dried in an oven at 50° C for one hour.

4.2.1.2 PURIFICATION OF FENFLURAMINE

Preheated ethanol (ca. 35° C) was added to the fenfluramine HCl crystals and heated gently until the crystals were dissolved. The resultant solution was filtered through a preheated Buchner funnel. Diethyl ether was added dropwise until the crystals started to form. At this stage the solution was placed in a cold room at 4° C to allow recrystallization to take place and the salt was isolated by suction filtration and dried in an oven at 50° C for 1 hour. The resultant dried white crystalline product had a melting point of 168.8° C.

The NMR spectrum of fenfluramine ($CDCl_3$) is shown in figure 6: δ 1.35 (3H, d, J 5,5 Hz, CH-C$\underline{H}_3$); 1,54 (3H, t, 5,6 Hz, $CH_2C\underline{H}_3$); 2,7-3,8 (6H, m, $PhC\underline{H}_2$-C$\underline{H}$-N$\underline{H}$-C$\underline{H}_2$); 7,2-7,6 (4H, m, ArH) ppm. The spectrometer was operated at 200 MHz and the chemical shift (δ) values were calculated downfield from trimethylsilane.

The IR spectrum (KBr) of fenfluramine is shown in figure 7: νmax (KBr) 3454 (NH); 1480 (C=C(aromatic ring)), 1338 (C=C(aromatic ring)), and 1166 (C-F) cm^{-1}. The I.R. spectrum was identical to the published spectra of fenfluramine[54].

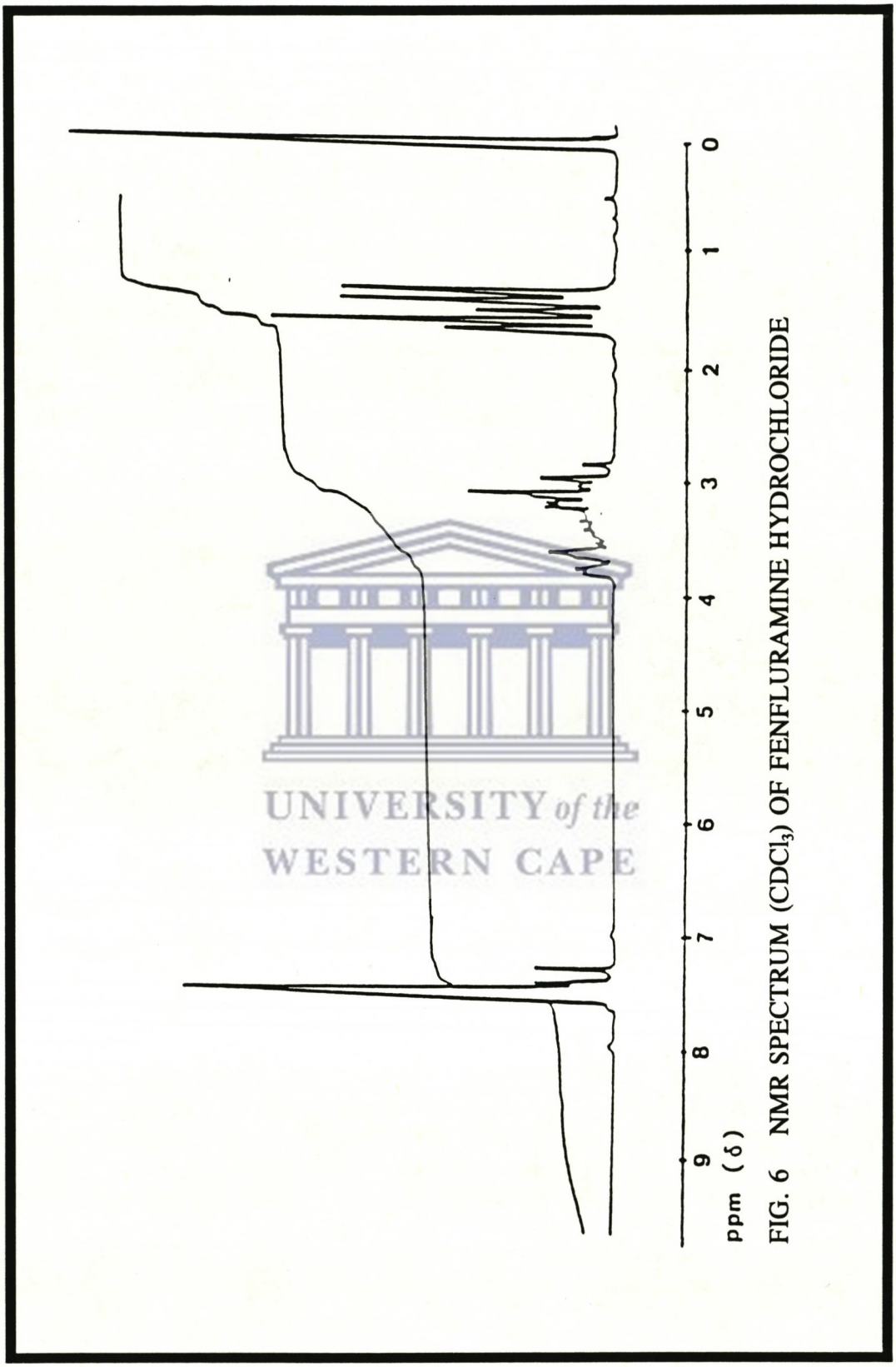


FIG. 6 NMR SPECTRUM ($CDCl_3$) OF FENFLURAMINE HYDROCHLORIDE

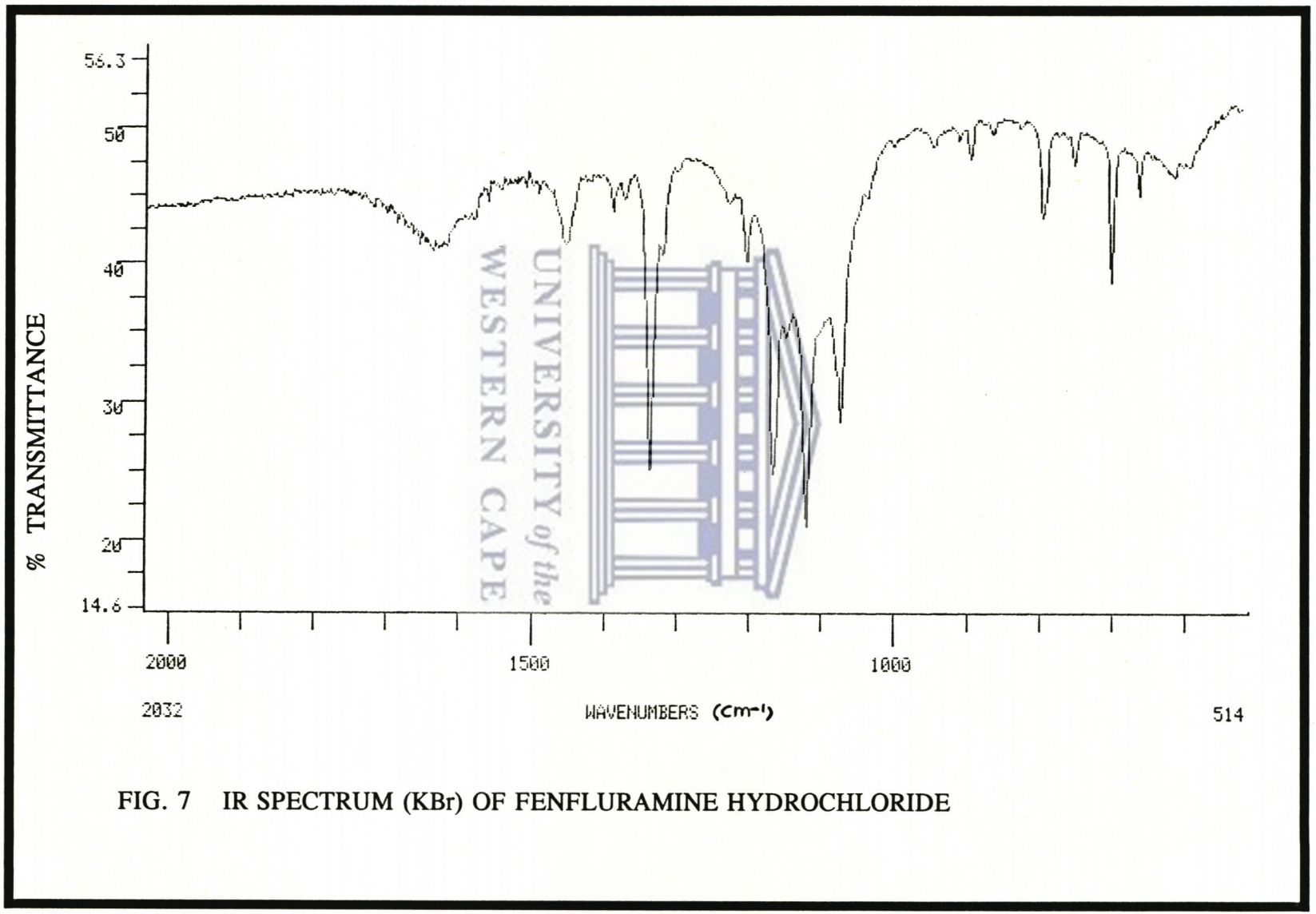


FIG. 7 IR SPECTRUM (KBr) OF FENFLURAMINE HYDROCHLORIDE

4.2.1.3 SYNTHESIS OF CHLORPHENTERMINE

Chlorphentermine hydrochloride was synthesized according to the procedure for clortermine hydrochloride, 1-(0-chlorophenyl)-2-methyl-2-aminopropane hydrochloride, given in the Pharmaceutical Manufacturing Encyclopedia[54]. The only difference between chlorphentermine and clortermine is that in the former compound the chlorine atom is in the para- instead of the ortho- position on the benzene ring. Thus for the synthesis of chlorphentermine, p-α-dichlorotoluene was used as the starting material instead of o-α-dichlorotoluene as for clortermine.

Eighteen grams of acetone was carefully added to the Grignard reagent prepared from 50.0 g of p-α-dichlorotoluene and 7.45 g of magnesium in diethyl ether. The reaction mixture was allowed to stand overnight at room temperature and was then carefully poured onto a mixture of 20 % sulphuric acid and ice. The organic layer was separated, washed with distilled water, an aqueous solution of sodium bicarbonate and again distilled water. The organic layer was subsequently dried over anhydrous magnesium sulphate and evaporated to dryness to yield an oily residue containing 1-(p-chlorophenyl)-2-methyl-2-propanol which was used without further purification.

To 29 ml of glacial acetic acid, cooled to 15° C, 11.5 g of sodium cyanide was added while being stirred. This was followed by the dropwise addition of 32,4 ml of concentrated sulphuric acid dissolved in 29 ml of glacial acetic acid while maintaining the temperature of the

reaction mixture at 20° C. The 1-(p-chlorophenyl)-2-methyl-2-propanol was added to the reaction mixture, allowing the temperature to rise. The resulting mixture was heated to 70° C while being constantly stirred. This mixture was then poured onto a mixture of water and ice and the resulting aqueous mixture was neutralised with sodium bicarbonate and extracted with three portions of diethyl ether. The combined ethereal extract was washed with distilled water, dried over anhydrous magnesium sulphate and evaporated to dryness. The resultant oily residue was mixed with 100 ml of 6 N aqueous hydrochloric acid and refluxed until a clear solution was obtained. Aqueous ammonia was added to the extract until the extract was alkaline (pH 9). It was again then extracted with three portions of diethyl ether. The combined ether extract was washed with distilled water and dried over anhydrous magnesium sulphate. Dry hydrochloric acid gas was bubbled through the ethereal solution and the hydrochloride salt of chlorphentermine which precipitated out of the solution was isolated by suction filtration and recrystalized from ethanol by the same method as described for fenfluramine (melting point 233.4° C).

The NMR spectrum ($CDCl_3$) of chlorphentermine is shown in figure 8: δ 1.4 (6H, s, $C\underline{H}_3C\text{-}C\underline{H}_3$); 3.0 (2H, s, $C\underline{H}_2$); 7.0-7.4 (6H, m, Ar$\underline{H}$, $N\underline{H}_2$) ppm. The spectrometer was operated at 200 MHz and the chemical shift (δ) values were calculated downfield from trimethylsilane.

The IR spectrum (KBr) of chlorphentermine is shown in figure 9: νmax 3486 (NH); 1492 (C=C(aromatic ring)), 1380 (C=C(aromatic ring)), 1049 (C-N stretch) and 820 (C-Cl) cm^{-1}. The I.R. spectrum was identical to the published spectra of chlorphentermine[54].

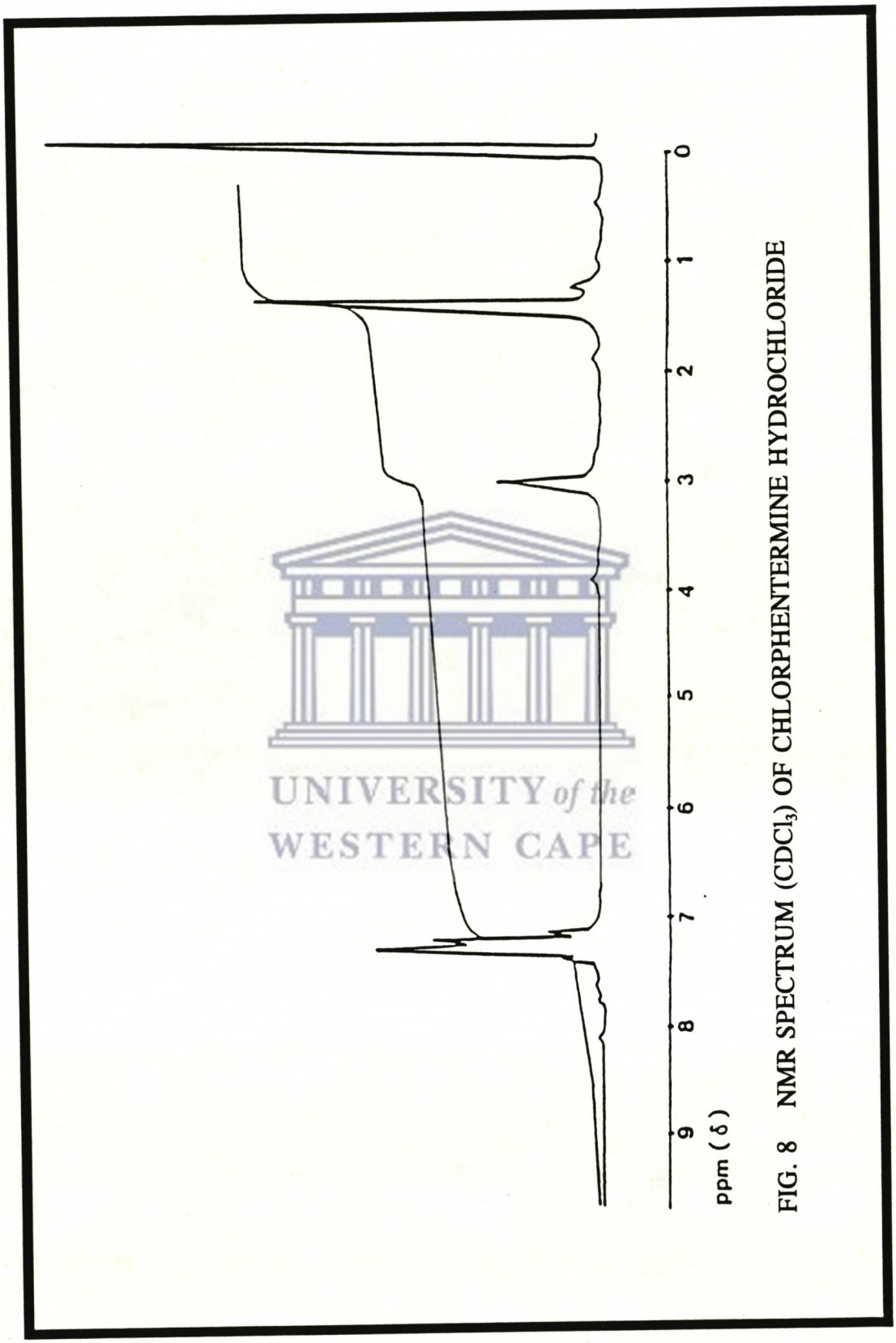


FIG. 8 NMR SPECTRUM ($CDCl_3$) OF CHLORPHENTERMINE HYDROCHLORIDE

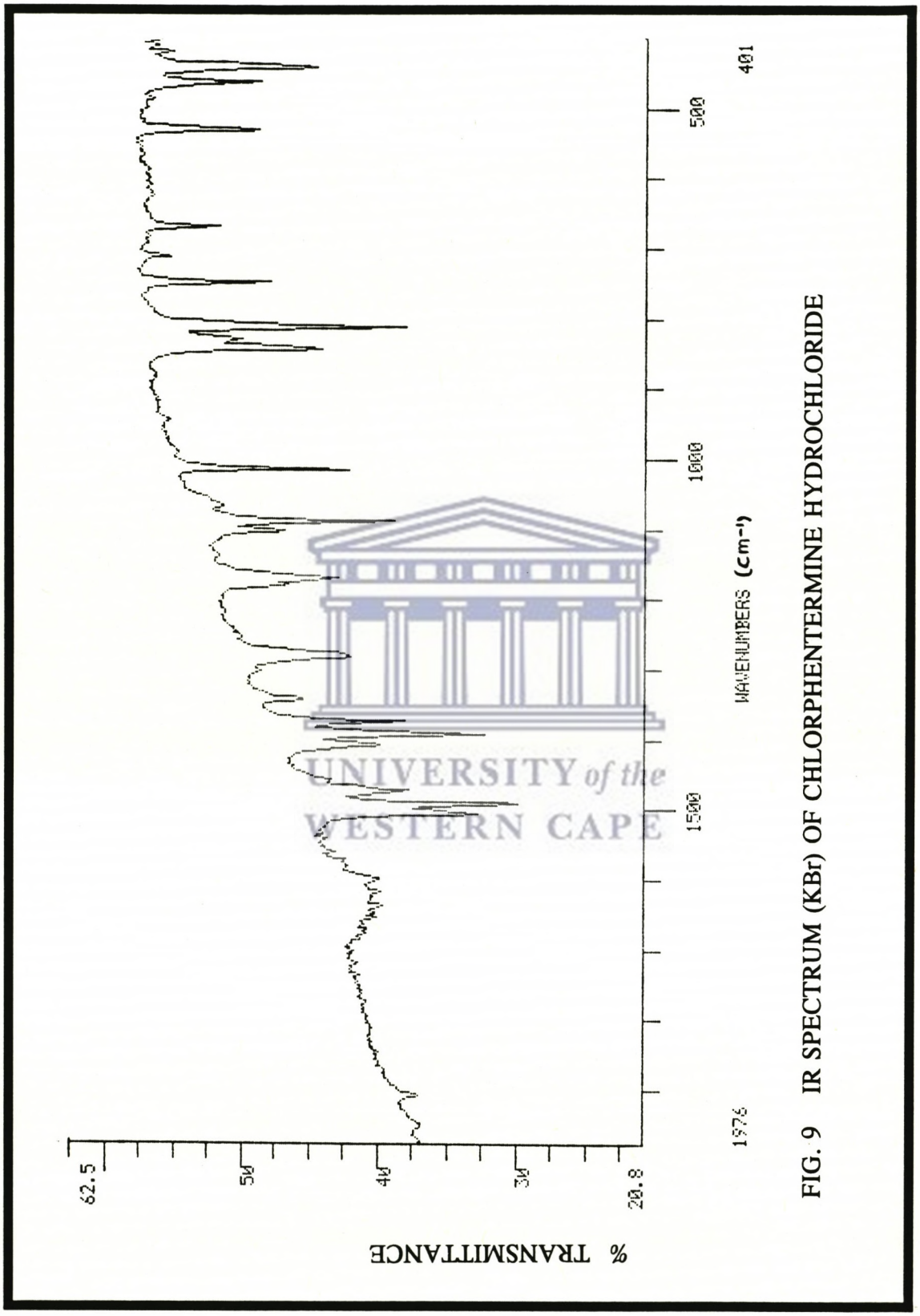


FIG. 9 IR SPECTRUM (KBr) OF CHLORPHENTERMINE HYDROCHLORIDE

4.2.2 INDUCTION OF PHOSPHOLIPIDOSIS

Wistar and BD9 rats, of both sexes, weighing ca. 300 g were used. All the animals were maintained on standard laboratory feed (i.e. Epol balanced rations) throughout the experiment, and had free access to water. Both the BD9 and Wistar rats were divided into groups of 8 per drug (i.e. chlorphentermine and fenfluramine) and saline controls. Throughout the treatment the lighting cycle of the animal room was controlled and set for ca. 10 hours bright light and ca. 14 hours dim light at room temperature (ca. 22° C).

4.2.2.1 DRUG ADMINISTRATION AND DOSING

During the dosing period the rats were weighed on Mondays, Wednesdays, and Fridays and their masses recorded. Chlorphentermine (CP) and fenfluramine (FF), reconstituted in physiological saline, were injected intraperitoneally (i.p.) for six days per week at a dose of 40 mg/kg body weight for 6 weeks[39,43]. The animals were injected intraperitoneally early in the morning (7h 00). Depending on their initial mass the control rats, both Wistar and BD9, received a corresponding volume of physiological saline.

4.2.3 ANALYSIS OF RAT LUNGS

From each group (ie. fenfluramine, chlorphentermine and saline) four rats were randomly selected and grouped, as indicated in the flowchart (see figure 10), for the isolation and analysis of,

(i) Unlavaged lungs and

(ii) Lungs lavaged to isolate macrophages

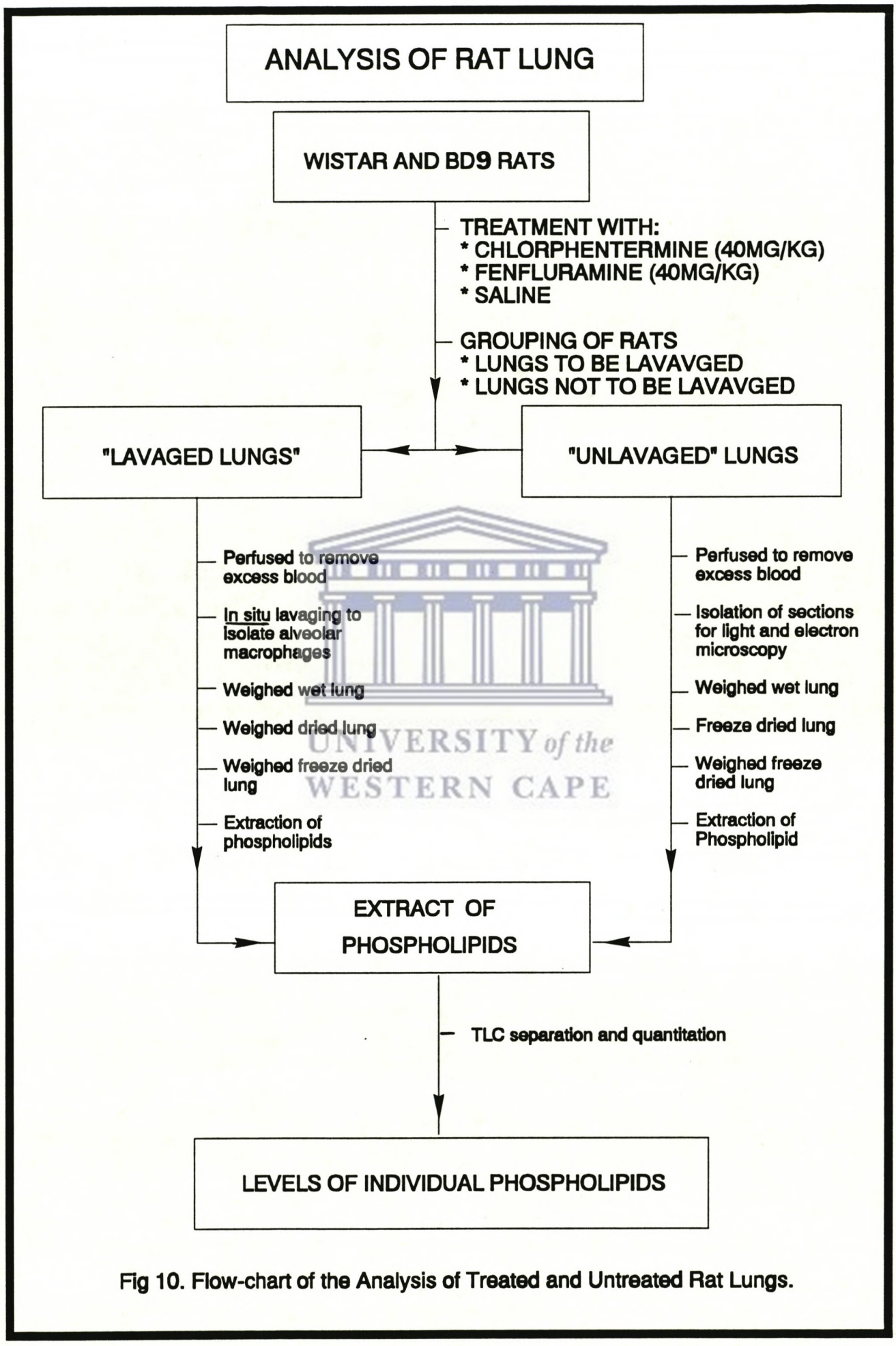


Fig 10. Flow-chart of the Analysis of Treated and Untreated Rat Lungs.

4.2.3.1 PROCEDURE FOR UNLAVAGED LUNGS

Twenty-four hours after the final injection of the drug or saline the animals were sacrificed under ether anesthesia. The chest was opened and the lungs perfused with 30 ml of saline introduced via the right ventricle after which the lungs were surgically removed. Excess blood vessels and connective tissue as well as the trachea were trimmed away. Two small portions of the middle lobe of the right lung were then used for the histological studies (see 4.2.3.1.1). The rest of the unlavaged lungs were then blotted dry with gauze, accurately weighed on a fine balance and cut into smaller pieces with a Mc-Ilvain tissue slicer. The slices were placed in a tarred glass tube, frozen on a dry ice-acetone bath and then freeze-dried to a constant weight. The freeze-dried lung was weighed and stored in an amber bottle in a vacuum dessicator, until further investigation.

4.2.3.1.1 HISTOLOGICAL STUDIES

The histological studies included both light microscopy and electron microscopy.

4.2.3.1.1.1 LIGHT MICROSCOPY

The aim of the light microscopic study was to observe any morphological changes which may have occurred in the lungs of the treated animals as compared to the lungs of the saline controls. For the sake of uniformity, two small portions of the middle lobe of the right lung were used for the histological examination.

4.2.3.1.1.1.2 FIXING OF LUNG PORTIONS

Each lung portion was fixed for 24 hours in 10 % v/v neutral buffered formalin (pH 7.0)[55]

Formula:[56]

Sodium dihydrogen phosphate (anhydrous)	3.5 g
Disodium hydrogen phosphate (anhydrous)	6.5 g
40 % w/v Formaldehyde	100 ml
Distilled water	900 ml

The specimen could remain in the fixative indefinitely.

4.2.3.1.1.1.3 PROCESSING PROCEDURE

After the specimen was fixed in 10 % neutral buffered formalin for two hours the specimen was dehydrated through exposure to a series of ascending grades of alcohol to remove all the water in the specimen. Thereafter the specimen was cleared in xylene and impregnated with molten paraffin wax. All this was done on an automatic tissue processor, the Histokinnette®. In each case the specimen was exposed to the following solutions for the times indicated:

10 %	Buffered formalin	2 hours
60 %	Alcohol	2 hours
70 %	Alcohol	2 hours
80 %	Alcohol	2 hours
90 %	Alcohol x 2	2 hours
100%	Alcohol x 2	1.5 hours
	Xylene x 2	2 hours
	Paraffin wax bath	2 hours

The specimens were then embedded in fresh paraffin wax, placed in a freezer for half an hour and allowed to set. Thereafter 3 μm sections were cut on a Universal sliding microtome, floated out on a waterbath, picked up with a slide and fixed in an incubator at 70° C for one hour. These sections were subsequently stained with haematoxylin and eosin, and Sudan IV[58].

4.2.3.1.1.1.4 SPECIAL STAINING OF LUNG SECTIONS

4.2.3.1.1.1.4.1 REAGENTS

The following reagents were used for the staining of the lung sections.

Mayer's acid alum haemotoxylin

Haemotoxylin is used to stain cell nuclei blue- black. The stain was prepared by dissolving 1 g of haemotoxylin and 50 g of potassium aluminium sulphate, ($KAl(SO_4)_2.12H_2O$), in one litre of

distilled water with the aid of gentle heat. Sodium iodate (0.2 g) was added to the latter solution and the mixture was left overnight at room temperature. The following day 50 g of chloral hydrate and 1 g of citric acid were added, the resulting mixture boiled for 5 minutes and then cooled to room temperature. The chloral hydrate was used as a preservative and citric acid to sharpen the nuclear staining. This mixture was allowed to stand for two weeks in order to mature[63].

Eosin

This is a red dye used to stain connective tissue and cytoplasm in varying proportions and shades of pink, orange and red and is used as a counter-stain stain. A 5 % stock solution was prepared in distilled water and a 1 % solution was used as the working solution[58].

Scott's tap water

This solution was used for the blueing of haemotoxylin. It was prepared by dissolving 3.5 g of sodium bicarbonate and 20 g of magnesium sulphate in one litre of tap water.

1% Acid alcohol solution

This solution was prepared by adding 20 ml of concentrated hydrochloric acid to 1.5 litre of 90 % alcohol and 480 ml of distilled water. The solution was used to differentiate the nuclei to

the desired degree.

Sudan IV

To prepare the stain 0.7 g of Sudan IV was dissolved in 100 ml propylene glycol solution (85 % in water), by adding small amounts at a time and the mixture heated to 100 - 110° C for ten minutes. Care was taken that the temperature of propylene glycol did not exceed 110° C since a gelatinous suspension would then form. The solution was filtered through Whatman No.2 paper, cooled and then filtered again through a fritted glass filter under vacuum.

4.2.3.1.1.1.4.2 PROCEDURE FOR HAEMOTOXYLIN AND EOSIN STAIN

The specimen slides were placed in a slide washing tray and dewaxed in xylene. They were then hydrated by passing them through a series of descending grades of alcohol to water. The slides were placed in Mayer's haemotoxylin and stained for ten minutes, removed, the excess haemotoxylin drained off and washed with tap water. Each section was decolourised by dipping it three times, for ten seconds each, into a 1 % acid alcohol solution followed by a water wash. Following this the slides were blued in Scott's tap water for 30 seconds, again washed with water and subsequently viewed under a low power staining microscope to inspect whether sufficient differentiation had occurred.

The specimen slides were counterstained by transferring the sections to 1 % eosin solution for 60 seconds, before being washed again with water. Thereafter they were dehydrated through a series of ascending grades of alcohol and cleared in xylene for fifteen seconds. The sections were tested for clarity by viewing them against a dark background with light striking them. Under these circumstances any patches containing water had a milky appearance and such sections were returned to absolute alcohol for a further 30 seconds. The fully prepared sections were mounted in Depex (DPX) and examined systematically under a double headed light microscope, at X10, X40 and X100 objective magnification.

The haemotoxylin and eosin stain was used to identify foam cells in the alveolar sacs and to observe any morphological changes of the lungs of rats treated with fenfluramine, chlorphentermine or saline[1].

4.2.3.1.1.1.4.3 PROCEDURE FOR SUDAN IV

Calcium was added to the formalin used to fix the lung portions for the Sudan IV stain to help contain phospholipids within cells.

The formal-calcium consisted of:

40 %	Formaldehyde	10 ml
10 %	Calcium chloride aqueous solution	10 ml
	Distilled water	80 ml

The specimens could remain in this fixative indefinitely. We further used the frozen section (cryostat) technique to avoid the extraction of fats from these tissue specimens which can occur when alcohol, paraffin solvents or heat is used.

Specifically, the tissue was placed on a moist blockholder, rapidly frozen with CO_2 and thereafter cut into 15 μm sections. The 15 μm frozen sections were washed in water for two minutes and then placed in propylene glycol solution twice for three minutes each time. The sections were stained for five minutes each in Sudan VI. They were then placed in 85 % propylene glycol for five minutes and washed in distilled water for five minutes. The sections were mounted on a slide in glycerine jelly and examined systematically under a double headed light microscope, at X10, X40 and X100 objective magnification.

This stain was used to confirm the increase in the total phospholipid concentration in the lungs, especially the alveolar macrophages of treated animals as compared to the saline controls.

4.2.3.1.1.2 ELECTRON MICROSCOPY

The electron microscope permitted the observation of cell and tissue structure beyond that seen with the light microscope. Both scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were used in this study.

4.2.3.1.1.2.1 REAGENTS

(a) MILLONIG'S BUFFER

41.5 ml 2.26 % $NaH_2PO_4.H_2O$

8.5 ml 2.52 % NaOH

The fixative was brought to pH 7.3 with 1 N NaOH.

(b) GLUTARALDEHYDE FIXATIVE

8 ml 25 % Glutaraldehyde

32 ml 2.26 % $NaH_2PO_4.H_2O$

The solution was brought to pH 7.3 with 1 N NaOH.

(c) OSMIUM TETROXIDE (OsO_4) FIXATIVE

The osmium tetroxide fixative was made up as follows: 45 ml of Millonig's buffer was added to an amber bottle, the label from the OsO_4 ampoule removed, and the ampoule scored in a fume cupboard. The entire ampoule with crystals was then placed into the bottle containing the buffer, the OsO_4 crystals were allowed to dissolve overnight and finally 5 ml of 5.4 % glucose solution added.

(d) SPURR'S RESIN

Spurr's resin was made up as follows:

ERL 4206	10 g
(Vinyl cyclohexane dioxide)	
DER 736	6 g
(Diglycidyl ether of propylene glycol)	
NSA	26 g
(Nonenyl succinic anhydride)	
S-1 catalyst	0.4 g
(Dimethyl aminoethanol)	

(e) PREPARATION OF REYNOLD'S LEAD CITRATE STAIN

1.33 g Lead nitrate

1.76 g Sodium citrate ($Na_3(C_6H_5O_2).2H_2O$

30 ml Distilled water (freshly boiled to remove carbon dioxide)

To a 100 ml beaker the lead nitrate, sodium citrate and 30 ml boiled distilled water was added, and the mixture allowed to stand for 30 minutes with intermittent shaking, to ensure complete conversion of lead nitrate to lead citrate. Thereafter the mixture was quantitatively transferred to a 50 ml volumetric flask, 8 ml of 1 N NaOH added, and made up to 50 ml with boiled distilled water and mixed by inversion[59].

4.2.3.1.1.2.2 PROCEDURES COMMON TO SEM AND TEM

For the sake of uniformity a small portion of the middle lobe of the right lung was used for electron microscopic examination. On excision of this portion of lung a piece of connective tissue was left on each specimen. The material was then handled or transferred by holding onto the connective tissue with a forceps without touching that part of the specimen that eventually was studied.

Immediately upon collection the specimen tissue was placed in glutaraldehyde fixative (pH 7.3) for twelve hours. Thereafter it was trimmed into smaller pieces with a razor blade. The cut tissue pieces were washed for thirty minutes in Millonig's buffer and post-fixed in osmium tetroxide (OsO_4) for one hour. The specimen was then washed again in Millonig's buffer for fifteen minutes and exposed through a series of ascending grades of alcohol for the following periods,

(1)	70 % Alcohol	15 minutes
(2)	90 % Alcohol	15 minutes
(3)	95 % Alcohol	15 minutes
(4)	Absolute alcohol	15 minutes
(5)	Absolute alcohol	30 minutes

It was now ready to be processed further for either SEM or TEM.

4.2.3.1.1.2.3 PROCEDURE FOR SEM

For SEM each specimen (ca. 2 mm thickness) was critical point dried, immediately stored in a dessicator and later, but soon afterwards, mounted and gold coated. The specimens were mounted by attaching them to stubs with the aid of double sided Scotch-tape. Since the tape is not conductive care was taken that it did not extend beyond the border of the stub. Also a dab of conductive cement (silver conductive paint) was applied to electrically connect the specimen to the metal of the stub.

Gold was used to coat the specimen surfaces. The gold was deposited slowly by means of a sputter coater to ensure thorough and even coating. The coated specimens were kept in a dessicator shielded from dust and vapours. Finally the stub with coated specimen was attached onto a mounting rod and inserted into the SEM port, the specimen located and the field searched for areas of interest. The latter was magnified X3 000, viewed and photographed.

4.2.3.1.1.2.4 PROCEDURE FOR TEM

Following the dehydration procedure described in 4.2.3.1.1.2.2, the specimens for TEM were further treated with,

(1)	Propylene oxide(P.O.) x 2	30 min
(2)	Spurr's resin + P.O.(1:1)	30 min
(3)	Spurr's resin	60 min
(4)	Spurr's resin	60 min

Strips of paper containing identification codes were placed in the Beem® capsules and then filled with Spurr's resin. The specimen was placed in the centre of the resin-filled capsule, and allowed to sink to the bottom. The capsules were placed in an oven at 70° C for 8 hours to polymerise.

Thereafter the Beem® capsule was removed, the area of interest

identified on the block and this area trimmed on the ultramicrotome in the form of a trapezium. The 1 μm sections were cut with specially made glass knives. The boat on the glass knife was filled with water, and the 1 μm sections were picked up with the aid of a bacteriological loop and placed onto a glass slide. The glass slide was heated gently with a spirits burner to evaporate off the water. Thereafter the specimen was stained with 1 % toluidine blue for five minutes and rinsed with distilled water. The resultant product was viewed under a light microscope. Upon confirmation that the area of interest had been isolated, ultrathin 70-80 nm sections were cut. The ultrathin sections were floated out in the boat, picked up onto grids, and stored in petri dishes.

4.2.3.1.1.2.5 PROCEDURE FOR DOUBLE STAINING OF ULTRATHIN SECTIONS

One drop of freshly prepared saturated uranyl acetate in 50 % alcohol was placed onto the specimen-containing grids for fifteen minutes at room temperature (ca.22° C). The grids were then immersed in 50 % alcohol for fifteen minutes and then in distilled water for a further fifteen minutes.

One drop of freshly prepared Reynold's lead citrate solution was placed onto the grids, for fifteen minutes at room temperature (ca. 22° C), before the grids were immersed in distilled water for one minute. One drop of 0.02 N NaOH was then placed onto the grids

and the grids left to stand for ten minutes before being rinsed with distilled water again. The stained grids were stored in an appropriately labelled petri dish, ready for viewing. To view, the stained grids were placed onto a mounting rod and inserted into the TEM port. Thereafter the specimen was located and the field searched for areas of interest. The latter were magnified X 10 000, viewed and photographed.

4.2.3.2 PROCEDURE FOR LAVAGED LUNGS

4.2.3.2.1 ISOLATION OF ALVEOLAR MACROPHAGES

Twenty four hours after the final injection of drug or saline, the animals were sacrificed under ether anesthesia and the lungs lavaged in situ. The trachea was sealed off, the chest opened and the lungs perfused with 30 ml of saline introduced via the right ventricle. The trachea was cannulated with polyethylene tubing which was connected via a spinal needle to a 20 ml syringe. The lungs were then lavaged with 10 ml aliqouts of warm (37° C), calcium- and magnesium- free Hank's balanced salt solution (pH ca. 7.6)(4.2.3.2.2) until 100 ml of the lavage fluid was collected. The latter lavage fluid was kept on ice until centrifugation.

4.2.3.2.2 HANK'S BALANCED SALT SOLUTION[37]:

MOLECULAR FORMULA	COMPONENT	MASS
NaCl	Sodium Chloride	8 000mg
KCl	Potassium Chloride	400mg
$Na_2HPO_4.2H_2O$	Sodium Phosphate	60mg
KH_2PO_4	Potassium Phosphate	60mg
	Anhydrous dextrose or Glucose	1 000mg
	Water to	100ml

To the above stock solution was added 10 ml of 0.2 % phenol red solution and 0.4 ml of chloroform and the resultant solution stored at room temperature. The final solution was made by diluting the stock solution 1 : 10 with distilled water and autoclaving 20 ml quantities together with 0.5 ml of 1.4 % sodium bicarbonate solution in McCartney bottles. The resultant solution was stored in a fridge with the caps partly unscrewed to allow equilibration at pH ca. 7.6.

4.2.3.2.3 ENUMERATION OF THE MACROPHAGES

An automatic electronic cell counter viz. the Coulter® counter, which operates on the principle that cells are poor conductors of electrical current, compared to saline solution, was used to enumerate the alveolar macrophages[60].

After collection of the lavage fluid the alveolar macrophages were recovered by centrifuging the lavage fluid at 500 g for ten minutes at a temperature of 4° C[61,62]. The supernatant was pipetted off and the pellet resuspended in 0.5 ml of physiological saline by vortexing for one minute. Forty microliters of the suspension was diluted to 2 ml with normal saline, and 0.5 ml aliqouts were passed through the counter. Three readings were recorded per fenfluramine, chlorphentermine and saline sample and the data recorded as number of macrophages/lung.

The data were analysed statisticaly using the Student's t-test for the grouped data and significant differences between mean values were indicated when the $P \leq 0.05$. After the alvoelar macrophages were isolated, the lavaged lungs were treated and freeze dried in exactly the same manner as the unlavaged lungs discussed earlier, and stored until further investigation.

4.2.3.3 PROCEDURES COMMON TO BOTH UNLAVAGED AND LAVAGED LUNGS

4.2.3.3.1 EXTRACTION OF LIPIDS

Throughout the procedures for the extraction of the lipids, the following precautionary measures were strictly followed:

(a) The use of plastic containers or apparatus, other than those made of Teflon was strictly avoided, as plasticisers could leach from them and might appear as peaks on the chromatograms.

(b) To prevent auto-oxidation of lipids samples were, wherever possible, handled in an atmosphere of nitrogen and an antioxidant e.g. butylated hydroxy toluene (BHT 50-100 mg/litre of solvent) was added to the extraction, thin layer chromatography and spray solvents.

(c) Before use solvents were also flushed with nitrogen to displace any dissolved oxygen.

(d) Both tissue and tissue extracts were always stored at -20° C under nitrogen[63].

4.2.3.3.1.1 EXTRACTION PROCEDURE:

Extraction of the lipid content from the lung tissue, was done according to the method of Radin[52]. The extraction solvent used was a mixture of hexane-isopropanol (HIP 3:2) v/v. To 100 mg of freeze-dried lung tissue 8 ml HIP (3:2) was added and the mixture vortexed for one minute and centrifuged at 6000 g for five minutes with a Beckman, Model TJ-6 centrifuge. Three milliliter of the supernatant was then transferred to a clean test tube. The lung tissue residue was re-extracted by adding 4 ml of HIP (3:2), vortexing for one minute and centrifuging at 6000 g for five minutes. Two milliliter of the supernatant was pipetted off and combined with the initial HIP (3:2) supernatant. The tissue residue was re-extracted two more times in this manner.

To remove small amounts of non-lipids in the HIP extracts, two milliliter "washing solution" was added to the combined supernatants. The "washing solution" consisted of 10 g anhydrous sodium sulphate in 150 ml water. The mixture was vortexed for one minute and centrifuged at 6000 g for two minutes. Eight milliliters of the supernatant was transferred to a tarred test tube.

The "washing solution" was back extracted with 3 ml of HIP (7:2) by vortexing for one minute and centrifuging at 6000 g for two minutes. Thereafter 2.5 ml HIP (7:2) was withdrawn and combined with the 8 ml HIP (3:2) and the combined extract evaporated under a stream of N_2 to a visually dry, oily residue with the aid of a Techne sample concentrator. The oily residue was weighed on a Mettler AE163 analytical balance, made up to 200 μl chloroform: methanol (2:1), subsequently transfered to an amber screw cap bottle and stored in a freezer at -20° C.

4.2.3.3.2 CHROMATOGRAPHY

The phospholipids were analysed by thin layer chromatography (TLC) using Merck 'high performance' silica gel precoated plates 20 x 20 cm, and 0.25 mm thick.

4.2.3.3.2.1 SOLVENT SYSTEMS

The phospholipids were separated by employing a two step one

dimensional procedure[64]. The two solvent systems used in this procedure were equilibrated in the developing tanks several hours before use and consisted of,

Solvent system A: Denaturated ethanol-chloroform-ammonium hydroxide (50:6:6. v/v/v) and

Solvent system B: Methyl acetate-n-propanol-chloroform-methanol-0.25 % aqueous potassium chloride (25:25:28:10:7 v/v/v/v/v).

4.2.3.3.2.2 SAMPLE APPLICATION AND CHROMATOGRAPHIC SEPARATION

The plates were prewashed in chloroform-methanol-water (60:35:8 v/v/v), up to the top of the plate, to remove interfering substances intrinsic to the plates before being dried for one hour at 110°C[65]. Ten microliter aliqouts of the samples and appropriate standards were applied to the plates and concentrated in a small spot by evaporating the solvent to dryness under a stream of nitrogen[66]. The samples were applied, at intervals of 15 mm on a baseline 10 mm from the bottom of the plate, and 10 mm from the side edge. The plates were developed in vertical chambers. Chamber one contained solvent system A, and the solvent front was allowed to migrate 30 mm from the baseline. Thereafter the plate was removed, the solvents evaporated with a hairdryer and the plate placed in an oven at 180° C for one minute to remove any residual solvents. The plate was

then cooled at room temperature (ca. 24° C) for 10 minutes and placed in chamber two which contained solvent system B. The solvent front was allowed to migrate till 10 mm from the top of the plate before the plate was removed, the solvents evaporated with a hairdryer and the residual solvents removed by placing the plate in an oven at 180° C for one minute. Again the plate was allowed to cool for 10 minutes. The spots were visualized by spraying the plate to transparency, with 10 % cupric sulphate in 8 % phosphoric acid[62], and then charring them at 180° C for 5 minutes. Each sample separation was done in duplicate.

4.2.3.3.2.3. DENSITOMETRY

The plates were scanned within 48 hours after charring, using a Shimadzu® CS 9000 densitometer. They were scanned in a horizontal mode parallel to the baseline using the position of the separated phospholipid standards as a guide for the scanning lane. Each lane was scanned three times and the average values of the three scans determined.

4.2.3.3.2.4 QUANTITATION OF PHOSPHOLIPIDS

In order to quantitate the separated phospholipids a standard curve of concentration ($\mu g/\mu l$) vs area was drawn for all the phospholipids. The concentrations spotted for the standards were 2 $\mu g/\mu l$, 5 $\mu g/\mu l$, 10 $\mu g/\mu l$ and 20 $\mu g/\mu l$. Each standard phospholipid was weighed and

made up to 200 μl with chloroform-methanol (2:1). Concentrations of the standards were spotted in duplicate and separated by a two step one dimensional procedure. Each lane representing the Y-axes was scanned three times.

The data for the lung samples and standard curves was analysed statistically using the Student's t-test for the grouped data and significant differences between mean $\pm$ standard deviation values were indicated when the $P \leq 0.05$. Each data point obtained is the mean of three readings.

4.2.4 ASSAY FOR PHOSPHOLIPASE ACTIVITY

Phospholipase A activity was measured by using Boehringer Mannheim test kits and the phospholipase C activity according to the method of Krug et al[67].

4.2.4.1 REAGENTS

(a) For phopholipase A activity determination

(1) Buffer solution:
Tris, 125 mmol/l, pH 8.0; $CaCl_2$, 4 mmol/l (bottle one).

(2) Phospholipase-A (PL-A) from porcine pancreas (bottle two).

(3) Stop reagent:
EDTA, 12 mmol/l; phosphate buffer, 50 mmol/l, pH 5.5 (bottle three).

(b) For free fatty acid determination

(1) Modified Dole's extraction medium:
isopropanol : heptane : 2 N sulphuric acid (40:10:1)

(2) Copper reagent:
7 ml triethanolamine, 0.3 ml glacial acetic acid, 3.25 g $Cu(NO_3)_2.3H_2O$, 6.25 g K_2SO_4, 17 g Na_2SO_4 and water to give a final volume of 100 ml.

(3) Sodium diethyldithiocarbamate: 0.1 % (w/v) in n-butanol

(4) 0.01 N sulphuric acid

(c) For phospholipase-C activity determination

(1) 0.4 M Tris-HCl, pH 7.3 at 37° C.
(2) 50 mM Calcium chloride ($CaCl_2$)
(3) 1 mg/ml Bovine serum albumin
(4) 0.2 M Ammonium sulphate
(5) 2 % Sodium dodecyl sulphate
(6) 1 mM Zinc chloride ($ZnCl_2$)
(7) One bottle containing alkaline phosphatase ≥ 100 U/ml and phospholipase-C ≥ 160 U/ml.

(d) For inorganic phosphate determination

(1) 0.6 M sulphuric acid (H_2S0_4)

(2) Molybdate solution:
2g of ammonium molybdate dissolved in 1 liter of H_2SO_4. Stable indefinitely.

(3) Tween 80:

One volume of Tween 80 mixed with two volumes of water.

(4) Working reagent:

100 ml of molybdate solution with mixed 0.9 ml of the diluted Tween 80. Use after 30 minutes.

(5) Stock phosphate standard 10 mg/100 ml 439 mg of KH_2PO_4 dissolved in water and dilute to 100 ml. Added a few drops of chloroform as a preservative.

(6) Working phosphate standard:

Diluted 5 ml of the stock standard to 100 ml with water.

4.2.4.2 PHOSPHOLIPASE-A ACTIVITY

PRINCIPLE

Phospholipids are hydrolysed to lyso-phospholipid and free fatty acid by phopholipase-A (PL-A). The formed free fatty acids can be measured quantitatively using a colorimetric assay[19]. The PL-A activity is calculated from the amount of fatty acids that are set free per unit of time.

4.2.4.3 PREPARATION OF SOLUTIONS

(a) Phospholipid micelles

The phospholipid micelles were prepared for each phospholipid by using

100 μl of phosphatidyl choline (PC), sphingomyelin (SPM), phosphatidyl ethanolamine (PE), phosphatidyl glycerol (PG), phoshatidyl serine (PS),phosphatidyl inositol (PI) or phosphatidic acid (PA) in chloroform:methanol (2:1) at a concentration of 5 μg/10 μl each and drying the sample under a stream of nitrogen. Thereafter ten microliters of Triton X-100 was added to each phospholipid sample and the mixtures were warmed to 40° C for 15 minutes and vortexed for 15 seconds, several times until a clear solution was obtained[68].

(b) PL-A stock solution

The PL-A stock solution was prepared by adding 3 ml of distilled water to the freeze dried PL-A, resulting in a final PL-A concentration of 12.5 U/l. Stable up to the expiry date specified, when stored in a fridge at + 4°C.

(c) The contents of bottles one and three (see 4.2.4.1 (a)) were used undiluted.

(d) Fenfluramine and chlorphentermine in distilled water were prepared at a concentration of 0.45 mM[21].

(e) Phospholipid-drug complex formation

The prepared phospholipid micelles (see 4.2.4.3 (a)) were each vortexed for 60 seconds with 300 μl of chlorphentermine or fenfluramine

solution, respectively, to allow for complex formation. The control phospholipid micelles were vortexed with 300 μl of distilled water.

(f) PL-A-drug complex formation

To 300μl of fenfluramine or chlorphentermine, 100μl of PL-A solution was added and vortexed for 60 seconds to allow for enzyme-drug complex formation.

4.2.4.4 HYDROLYSIS OF PHOSPHOLIPID

4.2.4.4.1 Protocol

The following protocol was used for the determination of PL-A activity viz. (i) phospholipase-A activity was determined in the presence of the phospholipid only, (ii) the phospholipid was shaken up with fenfluramine or chlorphentermine, allowing complex formation before PL-A was added, and (iii) fenfluramine or chlorphentermine shaken up with PL-A to allow for direct interaction between drug and enzyme, before the addition of the phospholipid.

4.2.4.4.2 PROCEDURE

All the experiments were conducted in a shaking waterbath at 37° C and in triplicate (see figure 11, flowchart). One hundred microliters of the PL-A solution were added to (a) the phospholipid micelles, previously

prepared (see 4.2.4.3 (a)), (b) the phospholipid micelle-fenfluramine complexes (see 4.2.4.3 (e)) or (c) the phospholipid micelle-chlorphentermine complexes (see 4.2.4.3 (e)), and then vortexed for 30 seconds and and the resulting reaction mixtures placed in a waterbath.

In the other series of experiments, to the previously prepared phospholipid micelles (see 4.2.4.3 (a)) we quantitatively transferred,(d) the PL-A-fenfluramine complexes (see 4.2.4.3 (f)) or (e) PL-A-chlorphentermine complexes (see 4.2.4.3 (f)), this was vortexed for 30 seconds and the resulting, reaction mixtures placed in a waterbath.

Once the various reaction mixtures were placed in the waterbath, the stopwatch was immediately started (time = 0) and after 30 minutes, 100 μl from each reaction mixture were transferred to test tubes containing 50 μl stop reagent, these tubes were vortexed for 30 seconds and placed in an ice bath at +4° C. These were then further analysed for the liberated fatty acids.

4.2.4.5 DETERMINATION OF THE LIBERATED FATTY ACIDS

Method

The liberated free fatty acids (FFA) were determined according to the method of Lauwerys[69]. The one hundred and fifty microliter of sample plus stop reagent were vortexed for 60 seconds with 4 ml modified Dole's extraction mixture. To this was added 6 ml heptane and 4 ml

water and the mixture vortexed again for 60 seconds. Five millilitres of the upper heptane layer were transferred to another test tube and vortexed for 60 seconds with 5 ml of 0.01 N sulphuric acid. Three millilitre of the upper heptane layer were transferred to a test tube containing 3 ml chloroform and 3 ml copper reagent, vortexed for 60 seconds and centrifuged at 800 g for ten minutes. Three millilitre aliquots of the upper chloroform-heptane phase were transferred to another test tube containing 0.5 ml diethyl-dithiocarbamate reagent. This was mixed and allowed to stand for 30 minutes and the absorbance was read at 440 nm against a reference of 3 ml chloroform:heptane (1:1) and 0.5 ml diethyldithiocarbamate reagent. The amount of liberated fatty acid was read off from the standard curve of the fatty acid concentration vs absorbance relationship (see 4.2.4.6).

4.2.4.6 QUANTITATION OF FATTY ACID CONCENTRATION, PL-A ACTIVITY AND % INHIBITION OF PL-A ACTIVITY

Palmitic acid was used as a standard to quantitate the fatty acids liberated by means of a standard curve of concentration ($\mu g/\mu l$) vs absorbance. The concentrations used for the standard curve were 2 $\mu g/\mu l$, 5 $\mu g/\mu l$, 10 $\mu g/\mu l$ and 20 $\mu g/\mu l$ and this was done in triplicate. The PL-A activity was calculated from the amount of free fatty acid set free per unit time, according to the following formula.

$$\text{PL-A activity } (\mu g \text{ FFA } 100\ \mu l^{-1} \text{ sample } min^{-1}) = \frac{x \mu g \text{ FFA } 100 \mu l^{-1} \text{ sample}}{30 \text{ min}}$$

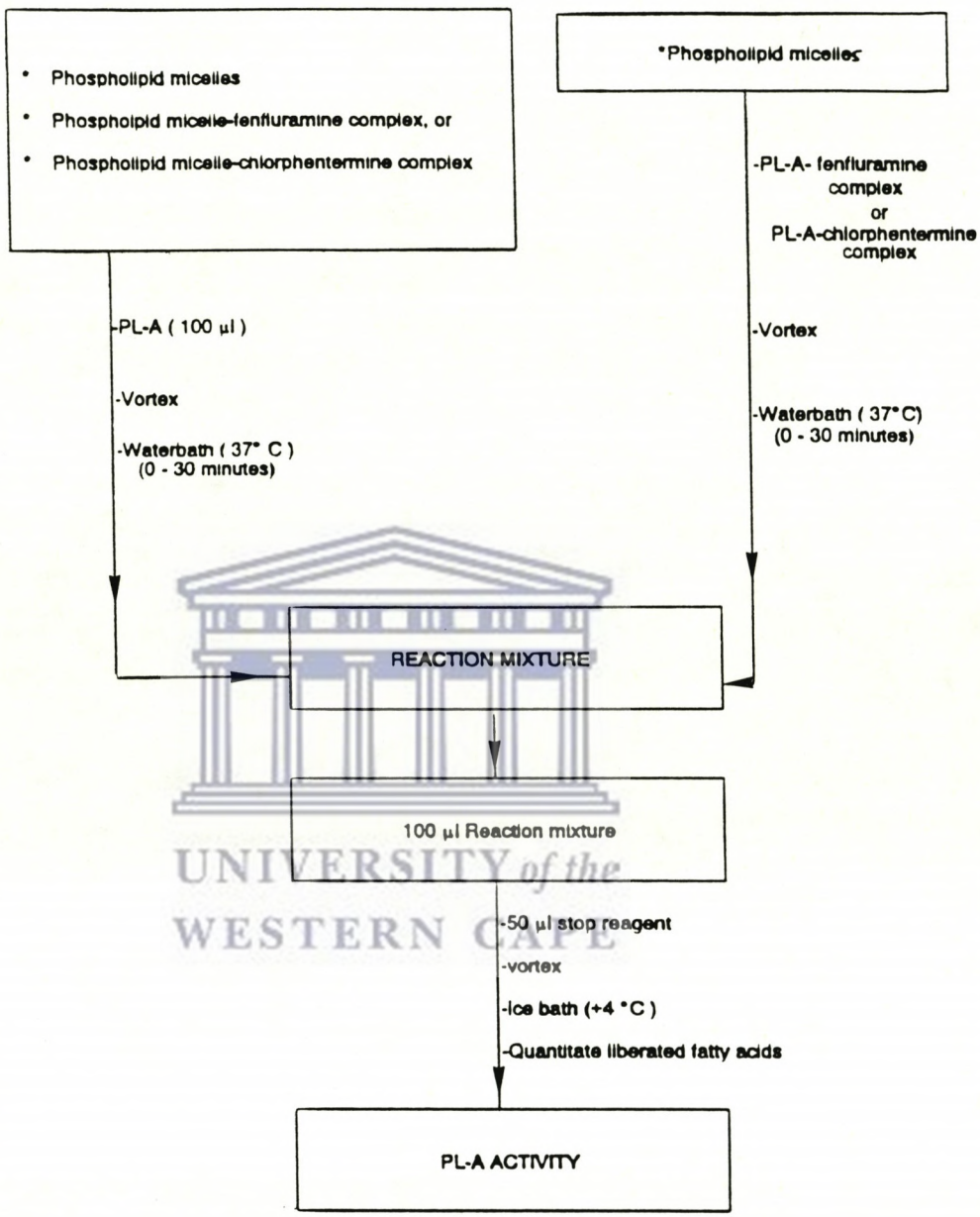


FIG. 11 FLOW CHART OF THE HYDROLYSIS OF PHOSPHOLIPIDS AND THE DETERMINATION OF PL-A ACTIVITY.

The percentage reduction in the PL-A activity, viz. % inhibition arising under the two test conditions i.e. phospholipid-drug complex and PL-A-drug complex was then calculated.

$$\% \text{ Inhibition} = \frac{\text{PL-A activity control - PL-A activity test}}{\text{PL-A activity control}} \times 100$$

The differences from the % inhibition of the different samples were analysed statistically using the Student's t- test for the grouped data and the level of significance set at $P \leq 0.05$. Each data point obtained is the mean of three readings.

4.2.4.7 PHOSPHOLIPASE-C ACTIVITY

PRINCIPLE

Phospholipids are hydrolysed to 1,2 diacylglycerol and a phosphobase by phospholipase-C (PL-C). The formed phosphobase can then be hydrolysed by alkaline phosphatase to liberate the inorganic phosphate and the latter detected colorimetrically. Finally the PL-C activity can be calculated from the amount of inorganic phosphate that are set free per unit time[67].

4.2.4.7.1 PREPARATION OF SOLUTIONS

(a) Phospholipid micelles were prepared as previously stated (see 4.2.4.3(a)).

(b) PL-C stock solution

The PL-C stock solution was used undiluted. It contained phospholipase-C ≥ 160 U/ml and alkaline phosphatase ≥ 100 U/ml. Stable up to the expiry date specified, when stored in a fridge at + 4° C.

(c) Fenfluramine and chlorphentermine in distilled water were prepared at a concentration of 0.45 mM[21].

(d) Phospholipid-drug complex formation.

The prepared phospholipid micelles i.e. PC, SPM, PI, PG, PS, PE and PA were vortexed for 60 seconds with 300 μl of fenfluramine and chlorphentermine solution, respectively, to allow for complex formation. The control phospholipid micelles were each vortexed with 300 μl of distilled water.

(e) PL-C-drug complex formation

To 300 μl of fenfluramine or chlorphentermine solution, 10 μl of PL-C solution was added and vortexed for 60 seconds to allow for complex formation.

4.2.4.8 HYDROLYSIS OF PHOSPHOLIPID

4.2.4.8.1 Protocol

The protocol used for the determination of PL-C activity was the same as that used for the PL-A activity (see 4.2.4.4)

4.2.4.8.2 Procedure

All the experiments were conducted in a shaking water bath at 37° C and in triplicate (see figure 12, flowchart). To (a) the phospholipid micelles, previously prepared (see 4.2.4.7.1 (a)), (b) the phospholipid micelle-fenfluramine complexes, (see 4.2.4.7.1. (d)) or (c) the phospholipid micelle-chlorphentermine complexes, (see 4.2.4.7.1. (d)) the following reagents were then added : 5 μl Tris-HCl, 52 μl calcium chloride, 40 μl zinc chloride, 53 μl bovine serum albumin and 144 μl ammonium sulphate. Ten microliters of PL-C stock solution (see 4.2.4.7.1 (b)) was then added to the above reaction mixtures, this was then vortexed for 30 seconds and placed in a water bath.

In the other series of experiments, to all the previously prepared phospholipid micelles (see 4.2.4.7.1 (a)) the same reagent mixture viz. 5 μl Tris-HCl, 52 μl calcium chloride, 40 μl zinc chloride, 53 μl bovine serum albumin and 144 μl ammonium sulphate was added. To the resulting mixtures, we quantitatively transferred (d) the PL-C-fenfluramine complexes (see 4.2.4.7.1 (e)) or (e) PL-C-chlorphentermine

complex (see 4.2.7.1 (e)), the tubes vortexed for 30 seconds and the mixture placed in a waterbath.

The stopwatch was immediately started (time = 0) and after 30 minutes the reaction was terminated by adding 200 μl of 2 % sodium dodecyl sulphate to the reaction vials[70]. This reaction mixture was then ready for further analysis.

4.2.4.9 DETERMINATION OF LIBERATED INORGANIC PHOSPHATE

Principle

The liberated inorganic phosphate was determined according to the method of Daly et al[71] which is based on the following principle: Phosphate reacts with molybdate in a strong acidic medium to form a complex. The absorbance of this complex in the near UV range is directly proportional to the phosphate concentration[72].

4.2.4.9.1 Method

To 10 μl microliters of each reaction mixture above, 1000 μl of working reagent (molybdate solution) (see 4.2.4.1 (d)(4)) was added. These reaction mixtures were then vortexed for 60 seconds at 22° C and the absorbance determined within 60 minutes at 340 nm using a Beckman DU40 spectrophotometer®.

4.2.4.9.2 Calculation of inorganic phosphorus concentration.

The absorbance (A) of the sample and standard (see 4.2.4.1 (d)(5)) was read against a reagent blank. The concentration of inorganic phosphorous was calculated from the following formula[71].

$$C(\mu g/100\ \mu l) = 10 \times \frac{\Delta A \text{ sample}}{\Delta A \text{ standard}}$$

The PL-C activity was calculated from the amount of inorganic phosphate (IP) set free per unit time according to the following formula:

$$\text{PL-C activity } (\mu g \text{ IP } 100\ \mu l^{-1} \text{ sample min}^{-1}) = \frac{x \mu g \text{ IP } 100 \mu l^{-1} \text{ sample}}{30 \text{ min}}$$

The percentage reduction in the PL-C activity i.e. % inhibition arising under the two test conditions i.e. phospholipid-drug complex and PL-C-drug complex was then calculated.

$$\% \text{ Inhibition} = \frac{\text{PL-C activity control - PL-C activity test}}{\text{PL-C activity control}} \times 100$$

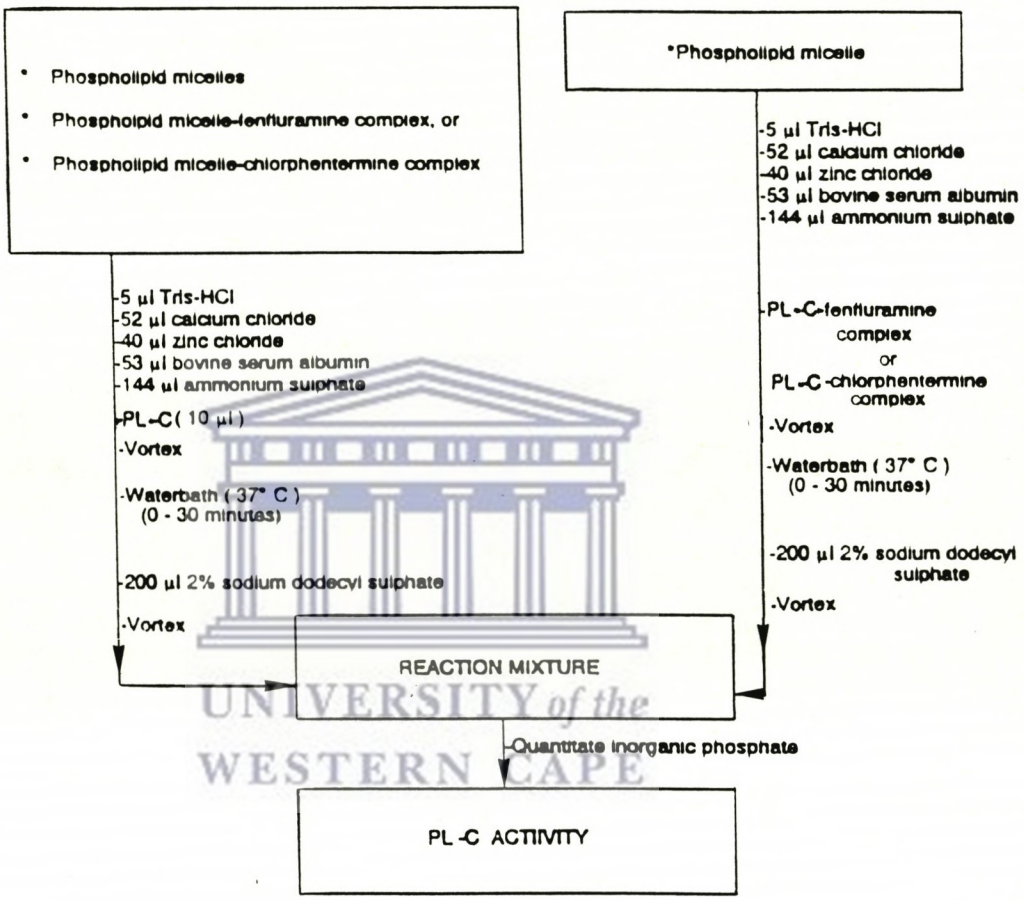


FIG. 12 FLOW CHART OF THE HYDROLYSIS OF PHOSPHOLIPIDS AND THE DETERMINATION OF PL-C ACTIVITY.

The differences in the % inhibition among the different samples were analysed statistically using the Student's t-test for the grouped data and level of significance was set at $p \leq 0.05$. Each data point obtained is the mean of three readings.

4.2.5 DIFFERENTIAL SCANNING CALORIMETRIC (DSC) DETERMINATION OF THE PHOSPHOLIPID PHASE TRANSITION TEMPERATURE

PRINCIPLE

Measurement of the differential power (heat input) necessary to keep a sample and a reference substance isothermal as temperature is changed (scanned) linearly is the basis of DSC. In DSC, the sample and a reference substance is subjected to a continously increasing temperature. The added heat compensates for that lost or gained as a result of endothermic or exothermic reactions occuring in the sample. A scanning calorimeter provides a convenient means for the precise determination of impurity limits in high-purity organic compounds, through observation of melting point depression. We wanted to use a DSC to measure the phase transition temperature of the phospholipid and phospholipid-drug complexes. It is postulated that the extent to which the phase transition temperature of the phospholipid is depressed by the drug (chlorphentermine or fenfluramine) may depend on the concentration of the drug within the phospholipid micelle, i.e. the extent of binding and/or on intrinsic ability of the intercalated drug molecules to perturb the arrangement of the adjacent phospholipids[73].

4.2.5.1 REAGENTS

14 mM Tris-HCl, pH 6.0 at 22° C.

4.2.5.2 PREPARATION OF SOLUTIONS

(a) Phospholipid micelles were prepared as previously stated (see 4.2.4.3 (a)).

(b) Fenfluramine and chlorphentermine in distilled water were prepared at a concentration of 0.45 mM[21]

(c) Phospholipid-drug complex formation

The prepared phospholipid micelles (see 4.2.4.3 (a)) were vortexed for 60 seconds with 300 μl of fenfluramine and chlorphentermine solution, respectively, to allow for complex formation. The control phospholipid micelles were each vortexed with 300 μl of distilled water.

4.2.5.3 DETERMINATION OF THE PHOSPHOLIPID PHASE TRANSITION TEMPERATURE (PTT)

4.2.5.3.1 Protocol

The following protocol was used for the determination of the phase transition temperature viz. (i) phase transition temperature was determined in the presence of the phospholipid only, (ii) the phospholipid was shaken up with fenfluramine or chlorphentermine, allowing complex formation before the phase transition temperature was determined.

4.2.5.3.2 Procedure

All the experiments were conducted in a shaking water bath at 50 °C and in triplicate. After evaporating the solvents under a stream of N_2 from (a) the phospholipid micelles, previously prepared (see 4.2.4.3 (a)), (b) the phospholipid micelle-fenfluramine complexes, (see 4.2.5.2 (c)) and (c) the phospholipid micelle-chlorphentermine complexes, (see 4.2.5.2 (c)) with the aid of a Techne S3® sample concentrator, the dry samples were resuspended in 100 µl of a 14 mM Tris-HCl buffer (adjusted to pH 6). Thereafter the phospholipid-micelles and phospholipid-micelle-drug complexes were constantly shaken for 2 hours. Every 30 minutes the samples were vortexed for 15 seconds to assure effective mixing.

4.2.5.3.3 Phase transition temperature measurement (°C)

The phase transition temperature (PTT) for each sample was determined with a Du Pont DSC® apparatus. The instrument was calibrated using an indium standard. Ten microliters of the above mixtures were transferred into an aluminium pan, frozen at -70 °C for 5 minutes and placed in the Du Pont DSC® apparatus, and which was measured against a blank aluminium pan containing 10 µl buffer only, also frozen at -70 °C for 5 minutes. Thermograms were established over a temperature range from 0 to 100 °C for (a) the phospholipid micelles (b) the phospholipid micelle-fenfluramine complexes and (c) the phospholipid micelle-chlorphentermine complexes, at a heating rate of 5° C min^{-1} and a sensitivity range of 0.5 mcal sec^{-1}.

4.2.5.4 ANALYSIS OF THE PHASE TRANSITION TEMPERATURE

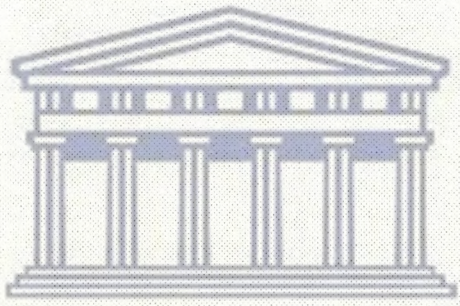
The phase transition temperature for the individual phospholipids and phospholipid-drug complexes were recorded in degrees centigrade (°C), and performed in triplicate. The data for the phase transition temperature were analysed statistically using the Student's t-test for the grouped data and the level of significant difference between the mean $\pm$ standard deviation values were indicated when $p \leq 0.05$. We then calculated the difference in the phase transition temperature (ΔT) from the values for the phospholipid control and phospholipid-drug complex, using the following formula,

$$\Delta T = \text{Phospholipid control} - \text{Phospholipid-drug complex}$$

as well as the relative percentage change in the phase transition temperature ($\Delta T\%$), using the following formula for the latter.

$$\Delta T\% = \frac{\Delta T}{\text{PTT of phospholipid control}} \times 100$$

CHAPTER FIVE



RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

5.1 BODY MASS AND FOOD INTAKE

The mean body mass for Wistar and BD9 rats recorded during and at the end of the experiment after chronic dosing with fenfluramine (40 mg/kg), chlorphentermine (40 mg/kg) or saline are shown in tables 1 and 2 and figures 13 and 14. Two of the initial 8 fenfluramine and chlorphentermine treated Wistar rats died after the first week of treatment, the rest survived till the end of the experiment. Three of the initial 8 chlorphentermine treated BD9 rats died after the first week of treatment, and one of the initial fenfluramine treated BD9 rats died after the first week of treatment. The dead rats in all instances were replaced, and new rats brought into the experiment. The mean body mass of the fenfluramine-treated Wistar (table 1) and BD9 (table 2) rats dropped rapidly at first and then remained relatively constant. The same applies to the chlorphentermine- treated Wistar and BD9 rats, the mean body mass dropped rapidly at first and then remained relatively constant throughout the period of treatment. The saline-treated Wistar and BD9 rats showed a steady increase in mass. After chronically dosing the rats with either fenfluramine, chlorphentermine or saline for six weeks, the mean final body mass of the Wistar rats treated with fenfluramine decreased by 17 g from the initial mean body mass while that of the chlorphentermine- and saline-treated rats increased by 6 g and 81 g respectively. The mean final body mass of the BD9 rats treated with fenfluramine or chlorphentermine decreased by 26 g and 48 g respectively while that of the saline-treated rats increased by 55 g.

TABLE 1: MASS OF WISTAR RATS AFTER CHRONIC ADMINISTRATION OF CHLORPHENTERMINE, FENFLURAMINE OR SALINE FOR SIX WEEKS. EACH MASS IS THE MEAN ± SD OF 8 RATS.

MASS OF WISTAR RATS (g)

DURATION OF TREATMENT (DAYS)	CHLORPHENTERMINE	FENFLURAMINE	SALINE
0	237 ± 55.06	274 ± 45.52	202 ± 61.50
6	231 ± 44.76	259 ± 46.77	242 ± 48.50
18	241 ± 32.96	256 ± 44.03	259 ± 41.16
27	243 ± 32.97	257 ± 43.95	270 ± 40.82
42	243 ± 32.40	257 ± 44.01	283 ± 39.73

TABLE 2: MASS OF BD9 RATS AFTER CHRONIC ADMINISTRATION OF CHLORPHENTERMINE, FENFLURAMINE OR SALINE FOR SIX WEEKS. EACH MASS IS THE MEAN ± SD OF 8 RATS.

MASS OF BD9 RATS (g)

DURATION OF TREATMENT (DAYS)	CHLORPHENTERMINE	FENFLURAMINE	SALINE
0	293 ± 51.07	345 ± 47.78	399 ± 89.43
6	258 ± 52.12	325 ± 46.98	426 ± 95.86
18	252 ± 49.97	321 ± 45.45	430 ± 84.86
27	246 ± 46.01	320 ± 45.27	442 ± 83.25
42	245 ± 45.77	319 ± 45.96	454 ± 84.97

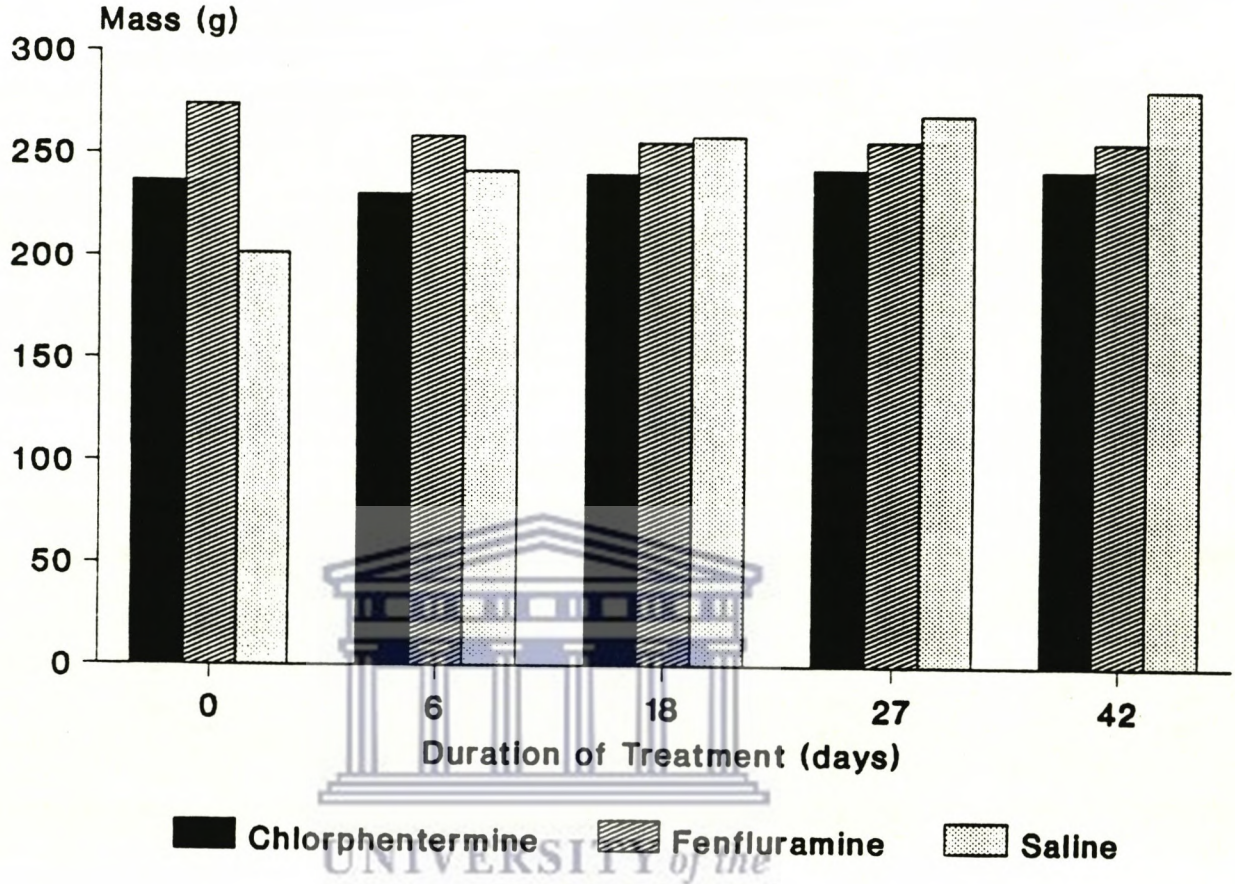


Figure 13. Bar graph of the mean body mass of Wistar rats after chronic administration of chlorphentermine, fenfluramine, or saline as a function of the duration of treatment.

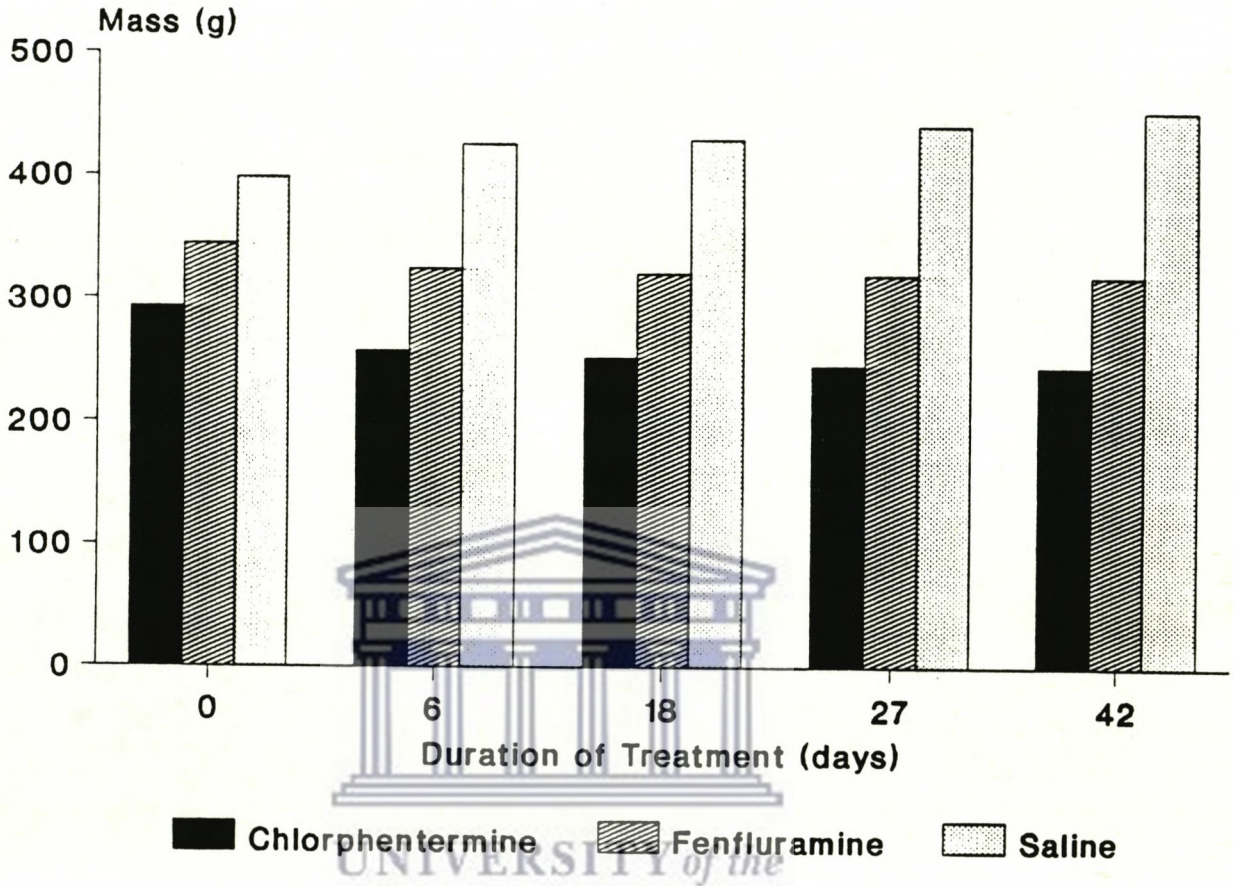


Figure 14. Bar graph of the mean body mass of BD9 rats after chronic administration of chlorphentermine, fenfluramine, or saline as a function of the duration of treatment.

In addition the administration of the drugs to the rat induced a behavioural syndrome of tremor and rigidity within three to five minutes following intraperitoneal injection. Trulson et al[74] reported the same effect after administration of 5-15 mg/kg doses of fenfluramine to rats. Their investigation revealed that this syndrome was a reflection of the potent stimulation of the postsynaptic serotonergic receptors by fenfluramine. These effects soon disappeared and the rats then became sedated. Tolerance, however, appeared to develop to these effects upon further administration of the drugs. The chlorphentermine- or fenfluramine-treated Wistar or BD9 rats became extremely agitated and aggressive and had frequent fights amongst themselves while the control rats did not behave in this manner.

Together the above results indicate that fenfluramine and chlorphentermine were effective anorexigenic agents in the rats at the dosage used in this study. Rodney et al[75] showed that chlorphentermine plasma concentration of 0.3 - 0.7 μg/ml produce anoretic effects in rats. Previous studies done in our laboratory has shown that at the dosage used, we expect plasma chlorphentermine levels of 3.8 - 6.4 μg/ml[39]. Chlorphentermine and fenfluramine also appeared to be more effective as anorexigenic agents in the BD9 rats compared to the Wistar rats.

5.2 IDENTIFICATION OF FOAM CELLS IN THE ALVEOLAR SACS

The results of the microscopic studies are shown in (figures 15 to 31). Light microscopic examination showed that after chronic administration of fenfluramine (n = 4) or chlorphentermine (n = 4) to the Wistar and BD9 rats, foam cells (figures 16 to 17) were present in almost all the alveolar sacs of the examined lungs of the rats treated with the latter drugs. Such foam cells were found in the lungs of all the chlorphentermine-and the fenfluramine-treated Wistar and BD9 rats. No foam cells were encountered

in the alveolar sacs of all the examined lungs of the control (saline-treated) rats (figure 15).

Structurally, the foam cells appeared to be oval, large and contained a bluish-purple stained nucleus. They were not evenly distributed throughout the lung tissue portions and in some sections they filled the entire alveolar sac whereas in others few foam cells were present or occured singly. The foam cells found in the lungs of the chlorphentermine- and fenfluramine-treated BD9 rats were more than those in the lungs of the Wistar rats.

The lungs of the Wistar and BD9 rats were also microscopically examined for their phospholipid distribution. The phospholipids in the lung stain blue-black after treatment with Sudan IV . The slides prepared from lungs of the fenfluramine- and chlorphentermine-treated Wistar and BD9 rats (figures 19 to 20) showed the presence of abundant phospholipids in the walls of the alveoli, the alveoli spaces and in the foam cells. No prominent evidence of phospholipid were evident in the walls of the alveoli, the alvoeli spaces and the macrophages isolated from the lungs of the control, saline-treated rats. (Fig. 18)

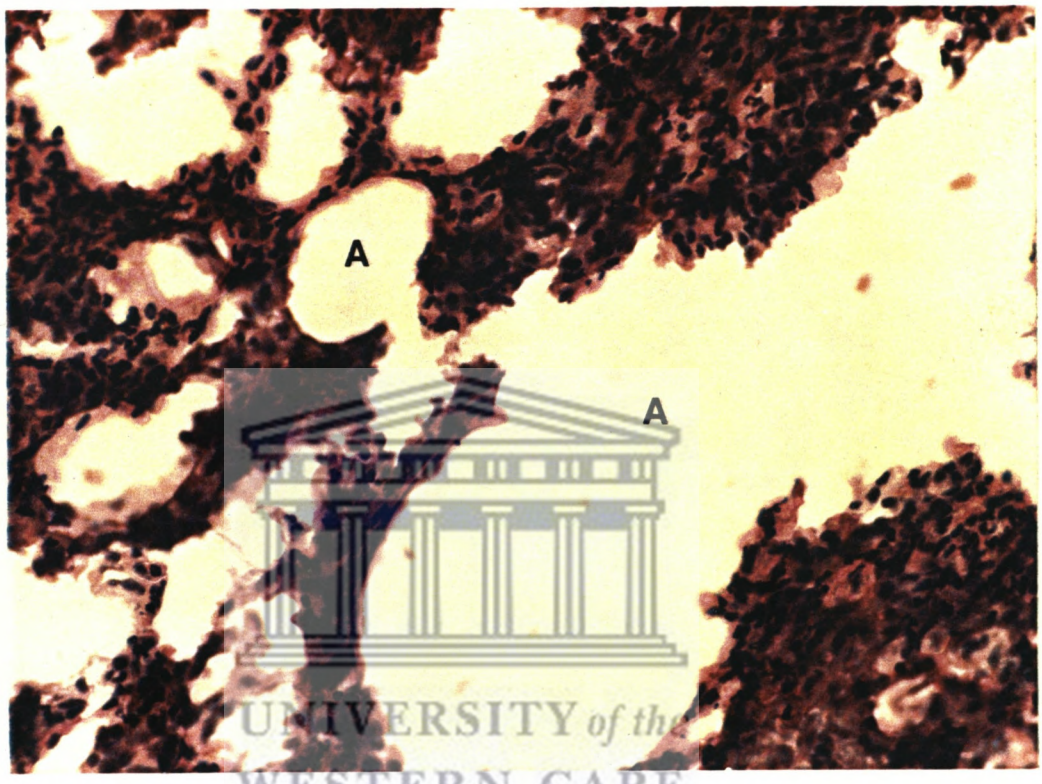
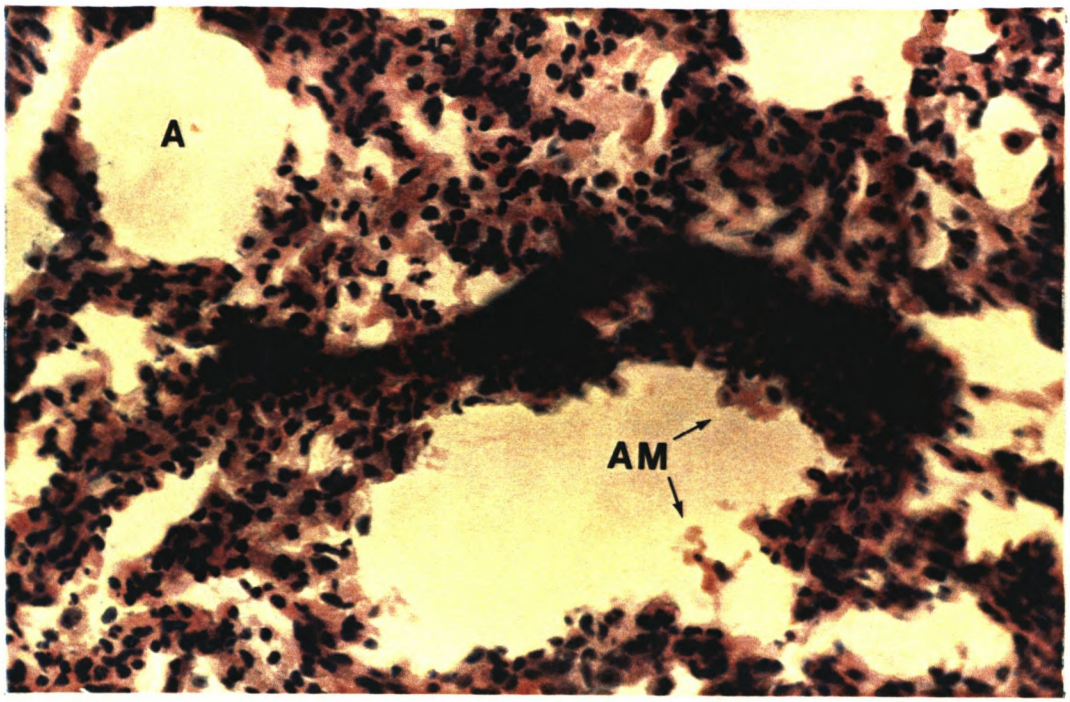


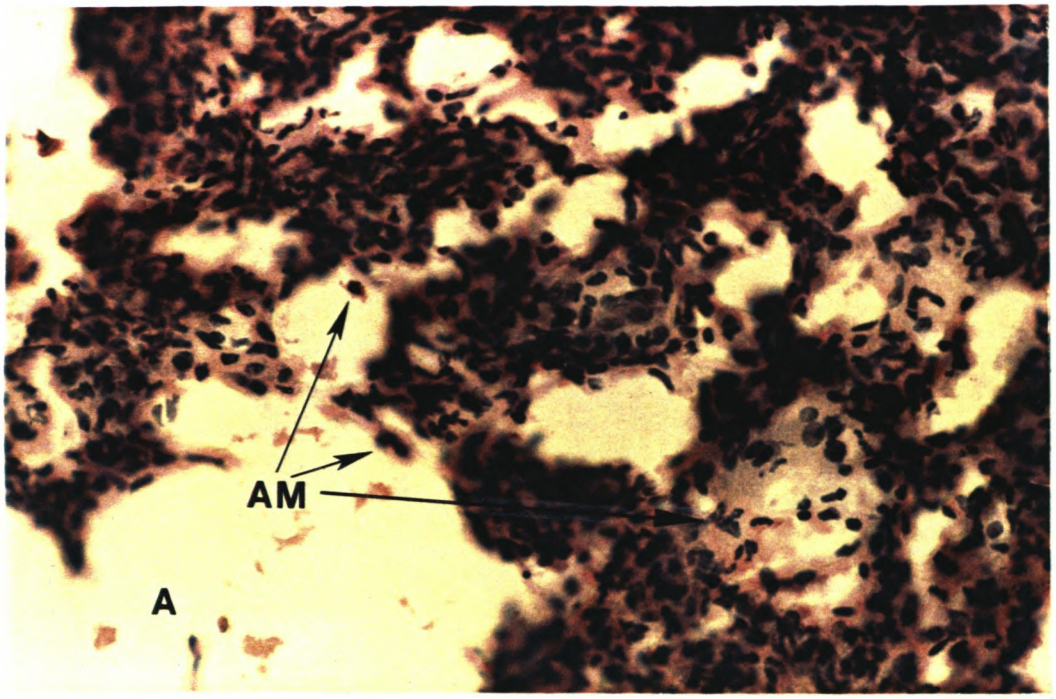
Fig 15. Lung section of control rats (Wistar or BD9) treated with saline for 6 weeks. Note that no foam cells are present in the alveolar spaces (A). Haemotoxylin and Eosin stain. Magnification 400X



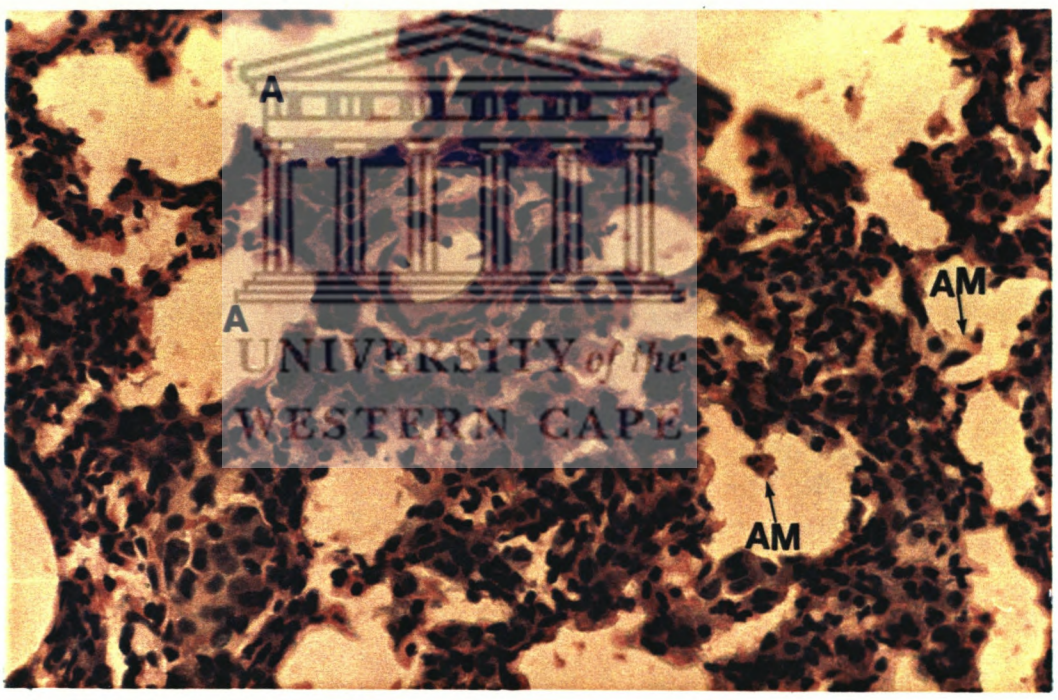
a

b

Fig 16. Alveolar macrophages (AM)"foam cells" in the alveolar spaces (A) of Wistar rats treated with either fenfluramine (a) or chlorphentermine (b) at a dose of 40mg/kg body weight for six weeks. Haemotoxylin and Eosin stain.
Magnification 400X



a



b

Fig 17. Alveolar macrophages (AM)"foam cells" in the alveolar spaces (A) of BD9 rats treated with either fenfluramine (a) or chlorphentermine (b) at a dose of 40mg/kg body weight for six weeks. Haemotoxylin and Eosin stain.
Magnification 400X

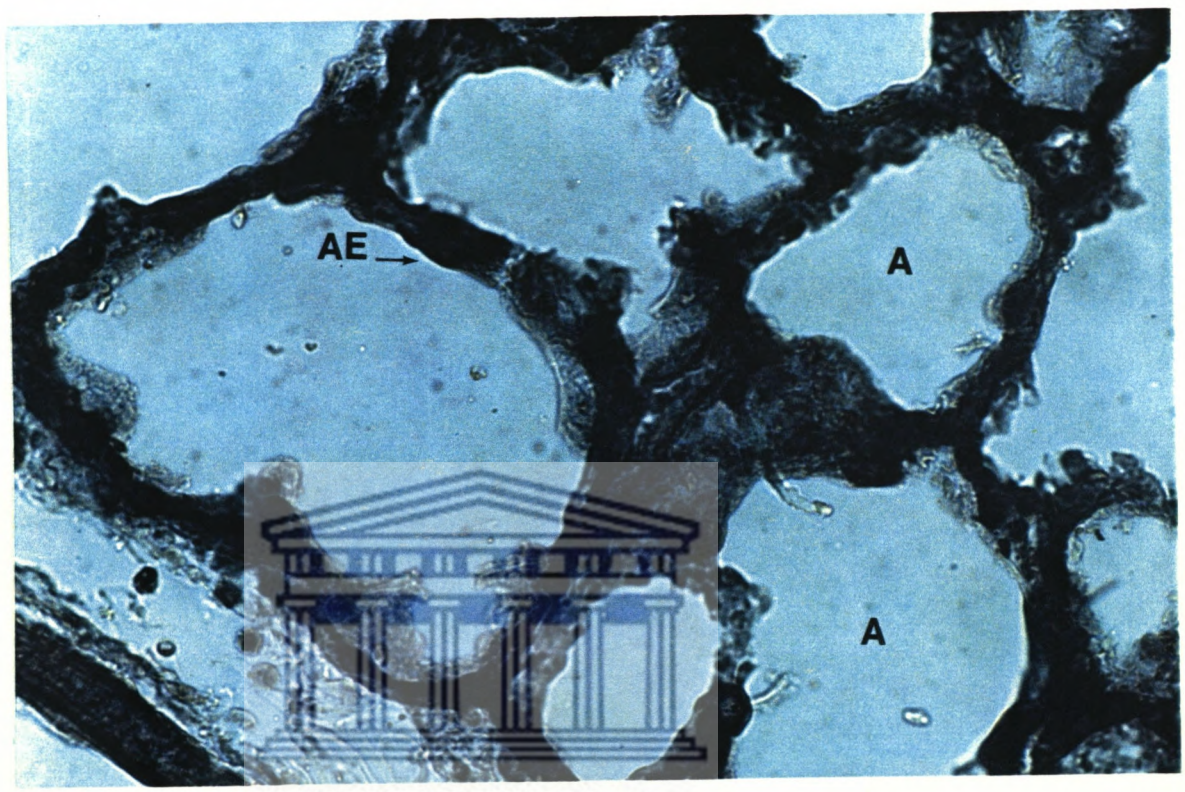
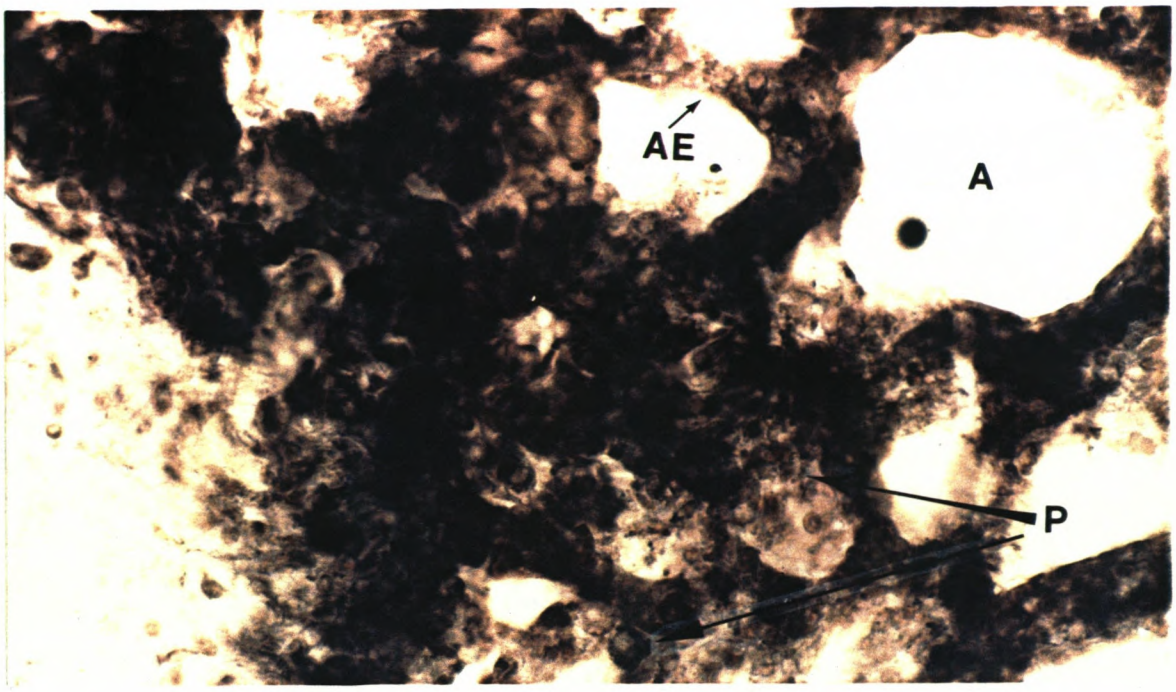
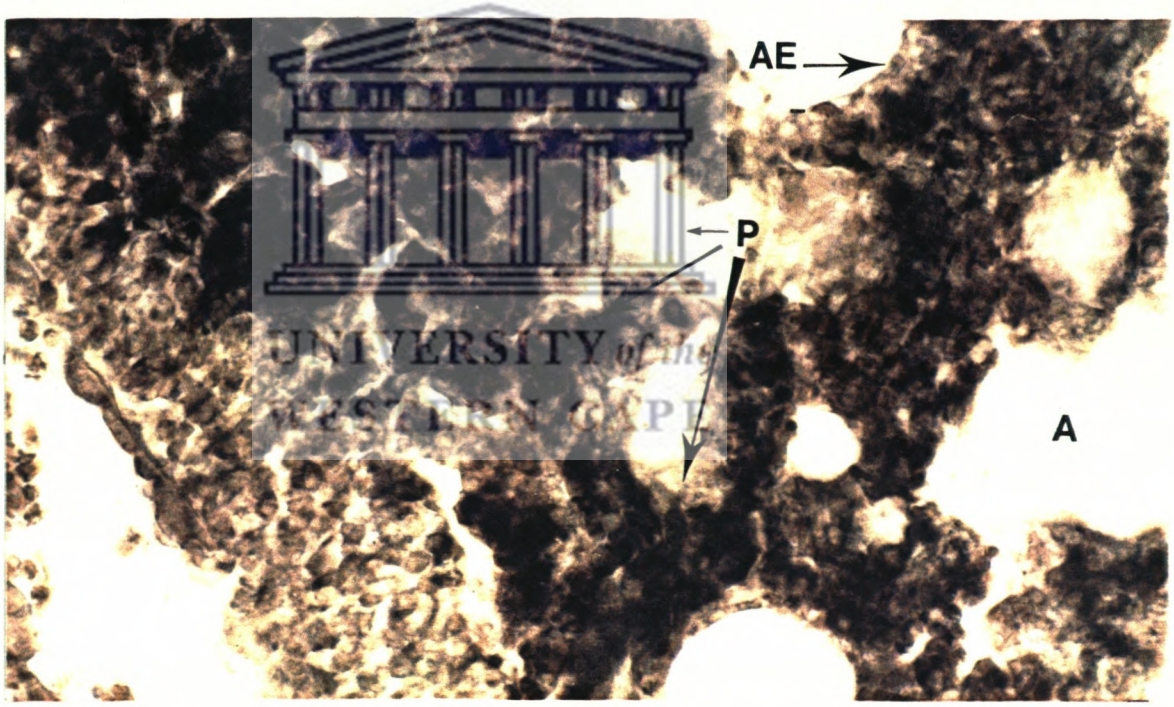


Fig 18. No prominent evidence of phospholipid distribution in the alveolar epithelia (AE) and alveolar spaces (A) of the saline control rats (Wistar or BD9) after six week treatment. Sudan IV stain. Magnification 1000X



a



b

Fig 19. Distribution of phospholipids (P) in the alveolar epithelia (AE), alveolar spaces (A) and alveolar macrophages (AM) of Wistar rats treated with fenfluramine (a) or chlorphentermine (b) at a dose of 40mg/kg body weight for six weeks. Sudan IV stain. Magnification 1000X

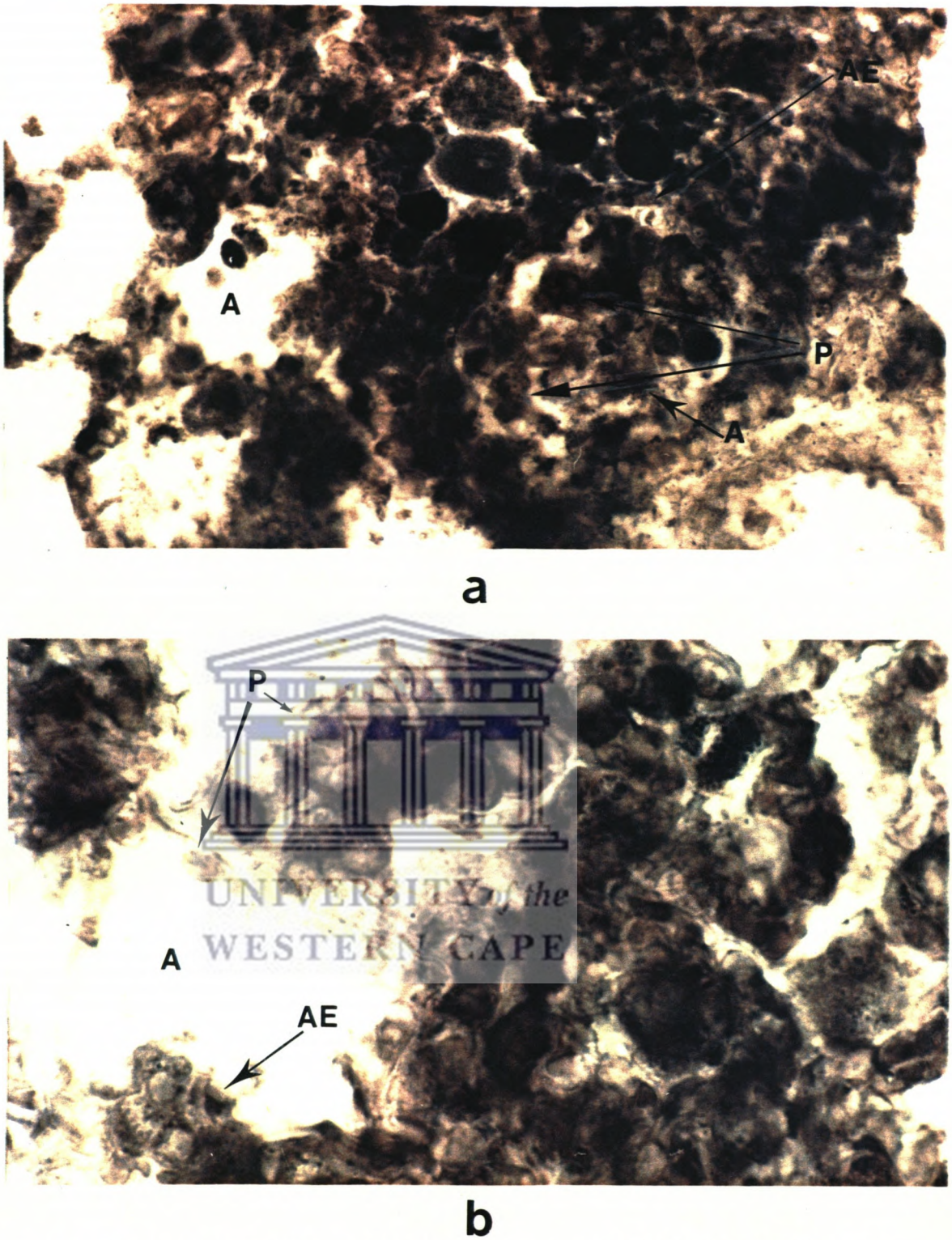


Fig 20. Distribution of phospholipids (P) in the alveolar epithelia (AE), alveolar spaces (A) and alveolar macrophages (AM) of BD9 rats treated with fenfluramine (a) or chlorphentermine (b) at a dose of 40mg/kg body weight for six weeks. Sudan IV stain. Magnification 1000X

Ultrastructurally, the scanning electron micrographs of the alveolar macrophages of fenfluramine- and chlorphentermine-treated Wistar and BD9 rats (figures 21 to 25), showed they had ruffled surface morphologies. This was also evident in the saline-treated Wistar and BD9 rats (figure 21). The transmission electron micrographs of the macrophages isolated from the fenfluramine- and chlorphentermine-treated Wistar and BD9 rats (figures 26 to 31) also showed that these cells contained prominant vacuoles, some of which were empty but others filled with concentrically arranged lamellar bodies. Fewer vacuoles and lamellar bodies were evident in the macrophages isolated from the saline-treated Wistar or BD9 rats (figure 26).

All in all the histological investigation of the lungs produced striking evidence of the presence of foam cells in the alveolar sacs of the fenfluramine- and chlorphentermine- treated Wistar and BD9 rats. These drugs thus induced pulmonary phospholipidosis in the Wistar and BD9 rats.

In a similar study Lüllman-Rauch et al[45] demonstrated that fenfluramine at a dosage of 40mg/kg/day induced foam cells in the lung alveoli of Sprague-Dawley rats. After treatment for two weeks, a few foam cells were observed, however, after four to six weeks of treatment they became more conspicuous. After ten weeks of treatment, the foam cells were found to be comparable in number to those seen in rats after six weeks of treatment, suggesting that a peak in the number of foam cells was reached by six weeks after initiation of therapy. The same study also revealed that guinea-pigs were less susceptible than rats to fenfluramine-induced phospholipidosis.

According to our study their is no evidence to indicate that fenfluramine or chlorphentermine treatment causes changes to the surface morphology of the alveolar macrophage compared to the saline treated cells. Takemura et al[76] observed that chronic exposure to inorganic dust e.g. coal, asbestos and silica caused surface alterations to the alveolar macrophage. These include increased rufflings, filopodia, pinocytotic vesicles and subplasmalemmal linear densities.

It has been repeatedly demonstrated that chlorphentermine induces foam cells in the alveolar sacs of rats. After chronic administration of chlorphentermine to the Sprague-Dawley rats, the increase in the lung to blood concentration ratio of chlorphentermine with time approximately parallelled the formation of cellular inclusion bodies and the increase in phospholipid content of the lung.[34,77] Kew and Narbaitz[78] observed that foam cells and large macrophages were present as early as 24 hours after the oral administration of 60 mg/kg body weight chlorphentermine to the Sprague-Dawley rat. Continuation of the regimen for 3 days increased the number of both free and attached hypertrophic macrophages within the pulmonary tissue. The foam cells increased progressively throughout the experimental period until a peak was reached by the first week after initiation of therapy. Continuation of therapy for a further 14 days did not produce a further augmentation in the number of foam cells. In another study, Reasor et al[4] treated Long Evans hooded rats with chlorphentermine for 4 weeks at a dose of 30 mg/kg intraperitoneally and examined the phospholipid content of the alveolar macrophages as a function of cell size. It was found that as the cells became larger the total phospholipid content increased proportionally. Thus the number and size of the foam cells are dependent on the dose of the administered drug and the duration of treatment. The interaction of chlorphentermine with the phospholipid is specific, since phentermine, differing from

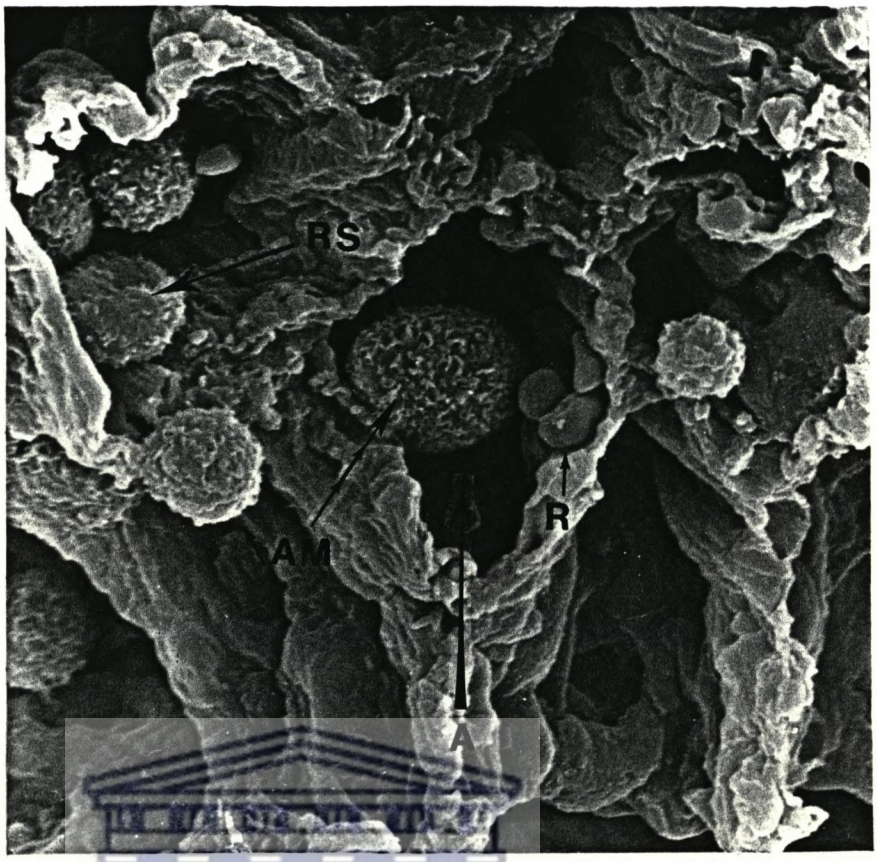


Fig 21. Scanning electron micrograph of an alveolar macrophage (AM) in the alveolar spaces (A) of control rats (Wistar or BD9) treated with saline for six weeks. The surface morphology shows ruffled surfaces (RS)°. Magnification 1500X

---

o R = Red blood cell

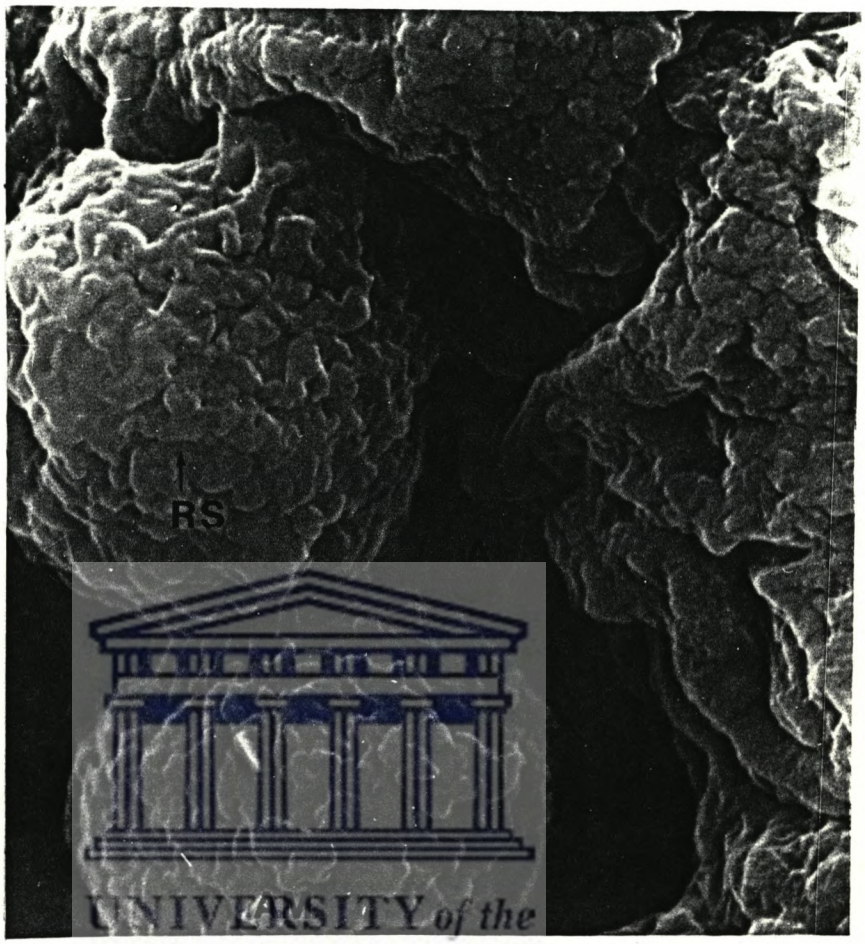


Fig 22. Scanning electron micrograph of alveolar macrophages (AM) "foam cells" in the alveolar spaces (A) of Wistar rats treated with fenfluramine at a dose of 40mg/kg body weight for six weeks. The surface morphology shows ruffled surfaces (RS). Magnification 7 000X.

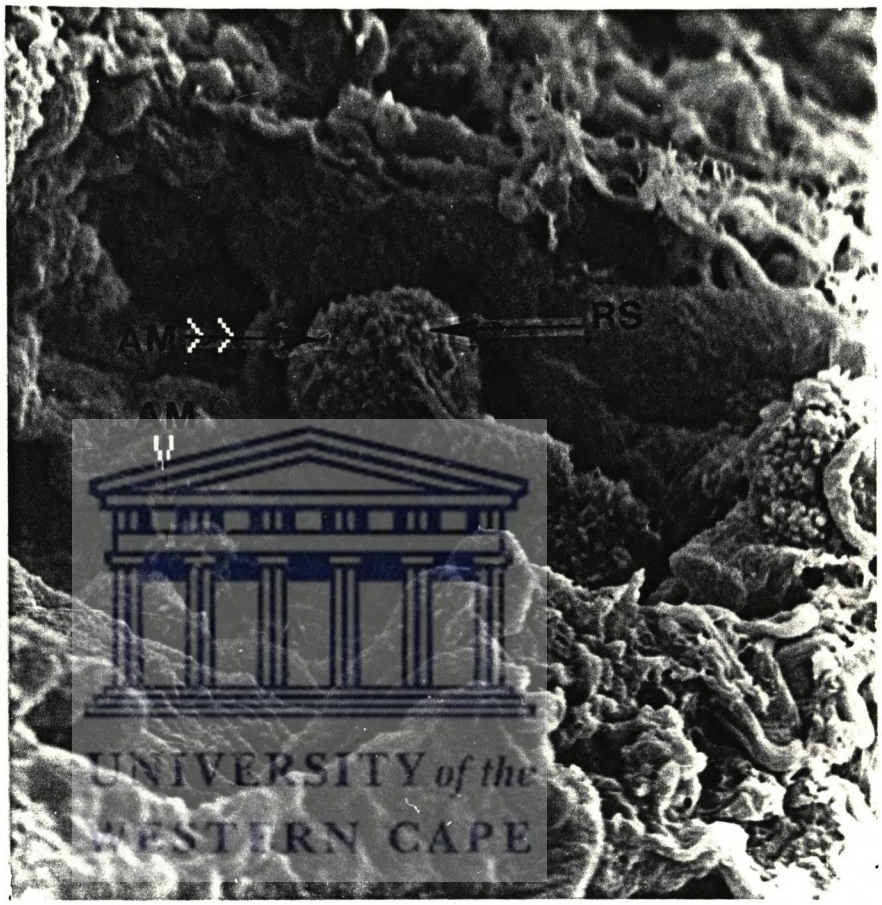


Fig 23. Scanning electron micrograph of alveolar macrophages (AM) "foam cells" in the alveolar spaces (A) of Wistar rats treated with chlorphentermine at a dose of 40mg/kg body weight for six weeks. The surface morphology shows ruffled surfaces (RS). Magnification 2 000X.

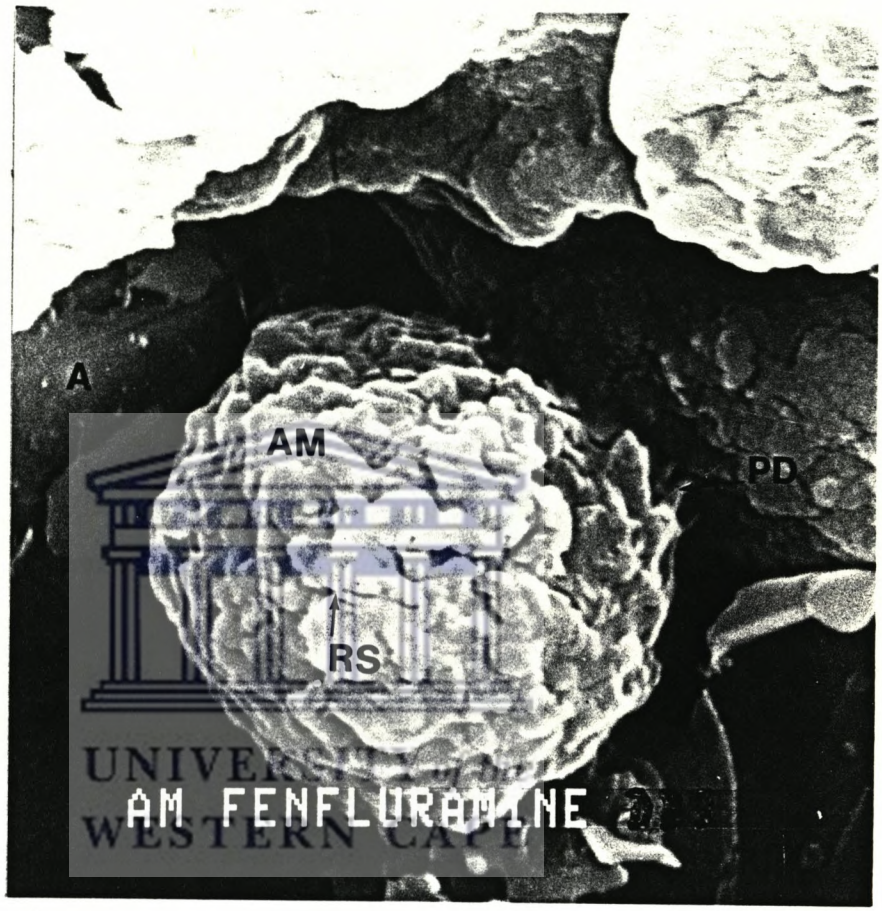


Fig 24. Scanning electron micrograph of alveolar macrophages (AM)[p] "foam cells" in the alveolar spaces (A) of BD9 rats treated with fenfluramine at a dose of 40mg/kg body weight for six weeks. The surface morphology shows ruffled surfaces (RS). Magnification 7 000X.

---

p PD = pseudopodia

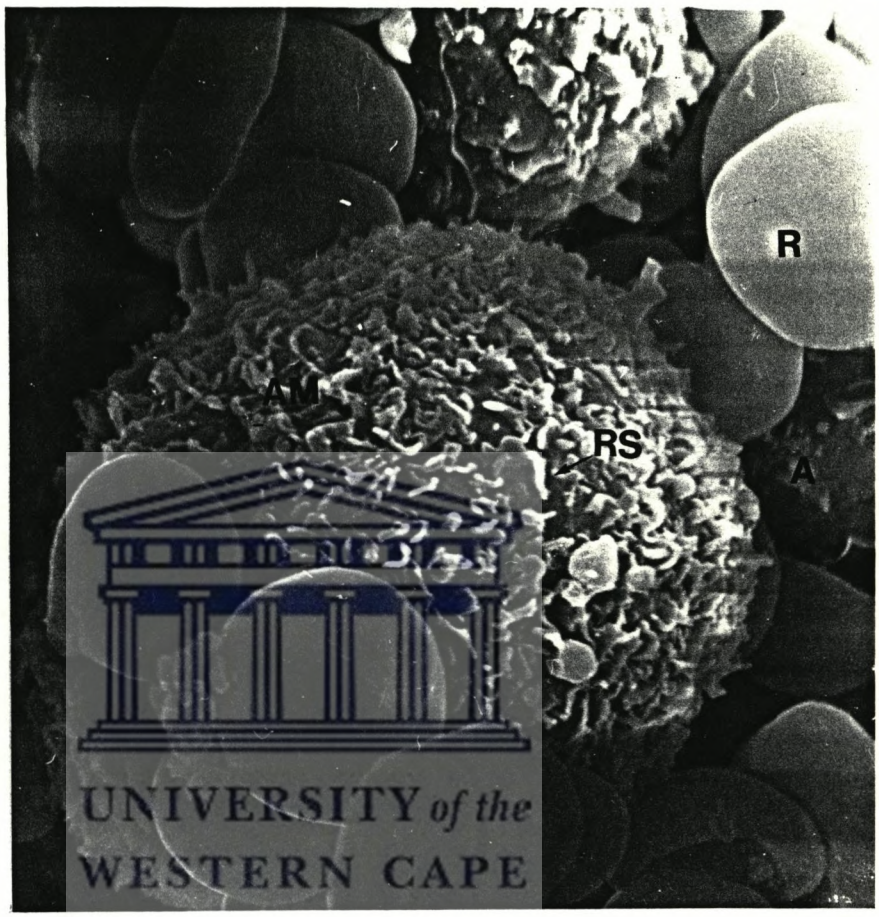


Fig 25. Scanning electron micrograph of alveolar macrophages (AM)[q] "foam cells" in the alveolar spaces (A) of BD9 rats treated with chlorphentermine at a dose of 40mg/kg body weight for six weeks. The surface morphology shows ruffled surfaces (RS). Magnification 5 000X.

---

q R = Red blood cell

Fig 26. Transmission electron micrograph of an alveolar macrophage (AM) of a control rat (Wistar or BD9) treated with saline. The alveolar macrophage is positioned on the blood-air barrier (B), is actively ingesting surfactant (S). Phagosomes are evident in the cytoplasm of the macrophage. Lamellar bodies (LB) are also evident. Magnification 9 000X.

Fig 27. Transmission electron micrograph of phospholipid rich lamellar bodies (LB), as it occurs in the alveolar macrophages.
Magnification 30 000X

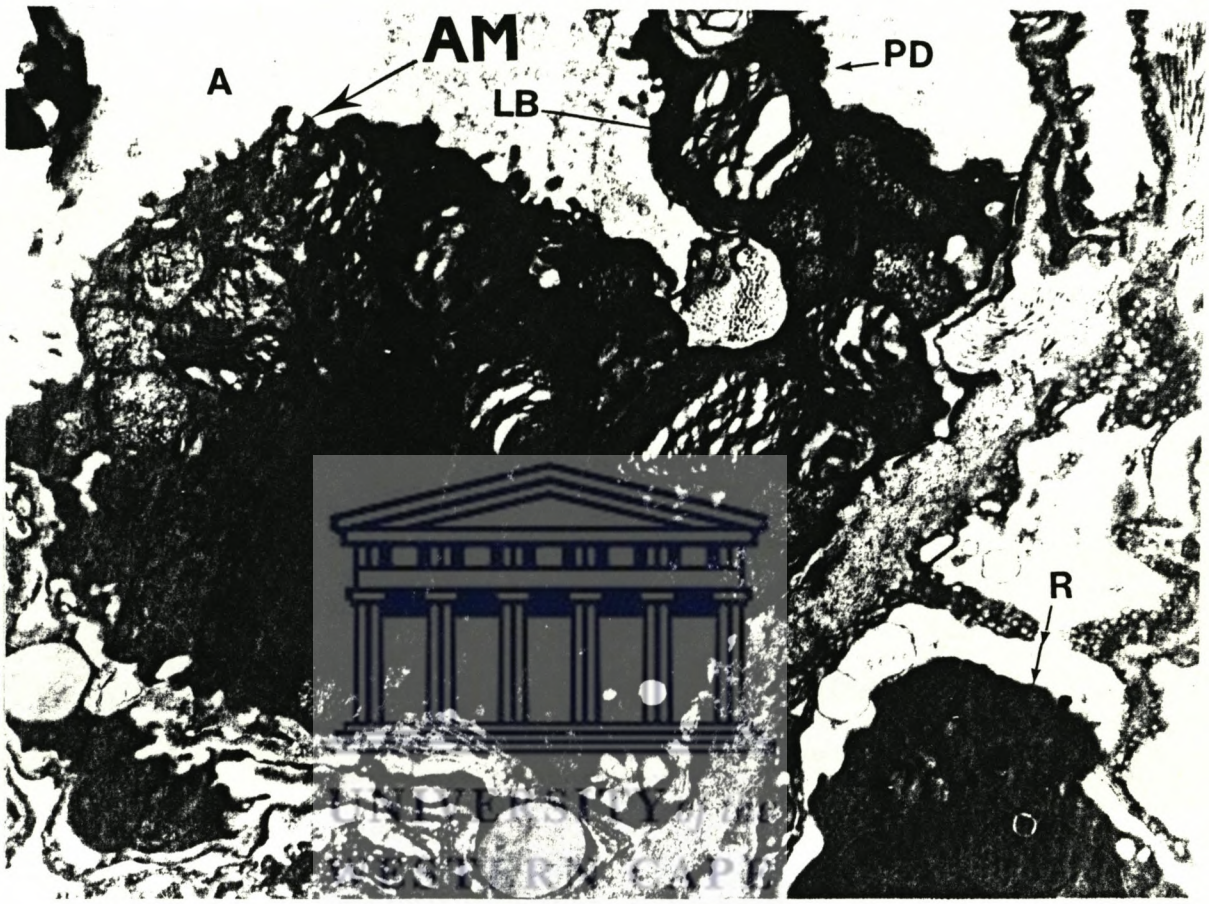


Fig 28. Transmission electron micrograph of an alveolar macrophage (AM)[r] "foam cell" in the alveolar spaces (A) of Wistar rats treated with fenfluramine at a dose of 40mg/kg body weight for six weeks. The foam cell contains many phospholipid rich lamellar bodies (LB). Magnification 8 000X

---

r PD = pseudopodia R = Red blood cell

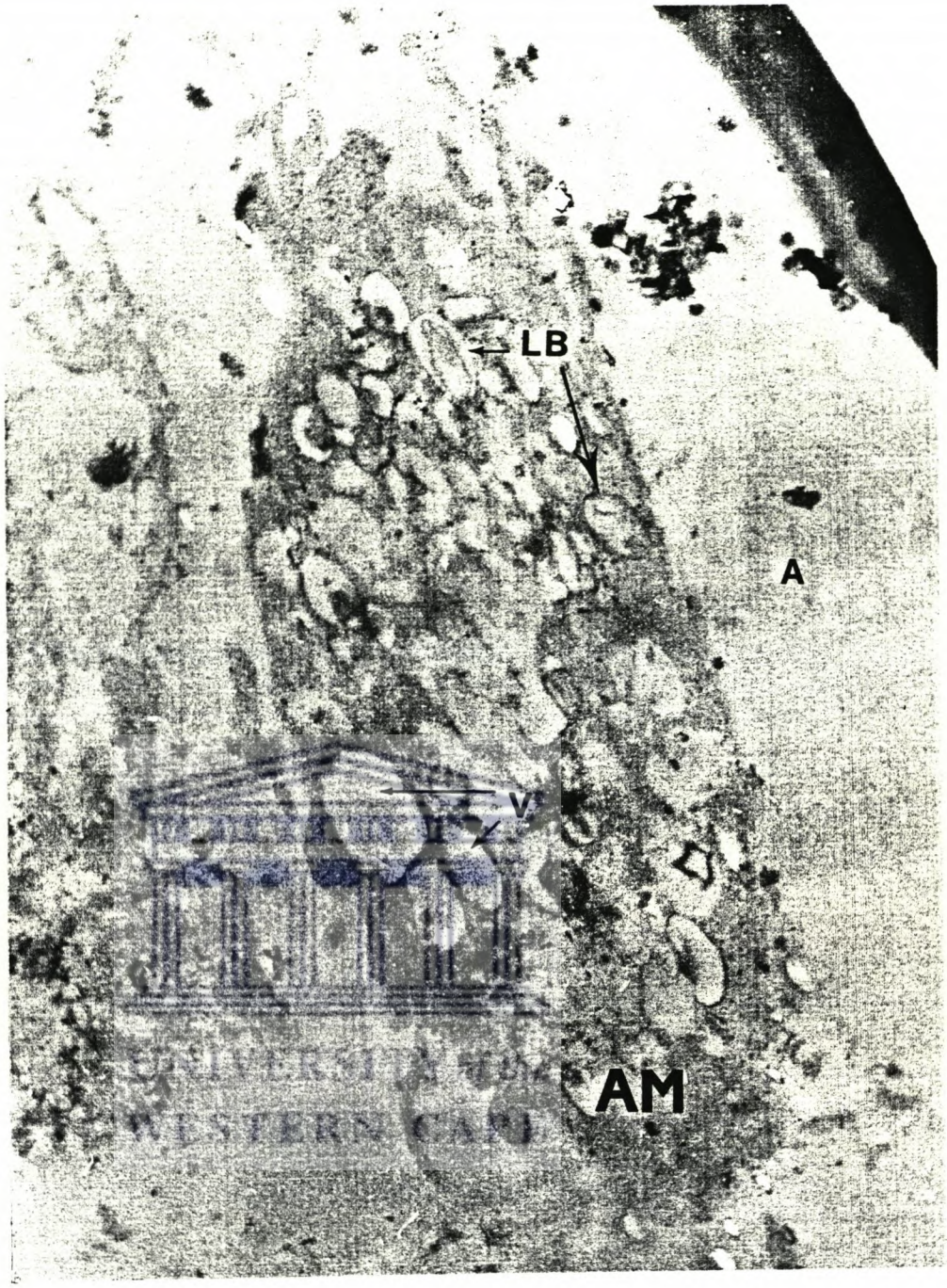


Fig 29. Transmission electron micrograph of an alveolar macrophage (AM) "foam cell" in the alveolar spaces (A) of Wistar rats treated with chlorphentermine at a dose of 40mg/kg body weight for six weeks. The foam cell contains many vacoules (V)[s].

Magnification 4 000X

---

s LB = Lamellar body

Fig 30. Transmission electron micrograph of an alveolar macrophage (AM)[t] "foam cell" in the alveolar spaces (A) of BD9 rats treated with fenfluramine at a dose of 40mg/kg body weight for six weeks. The foam cell contains phospholipid rich lamellar bodies (LB). Magnification 5 000X

---

t PD = pseudopodia

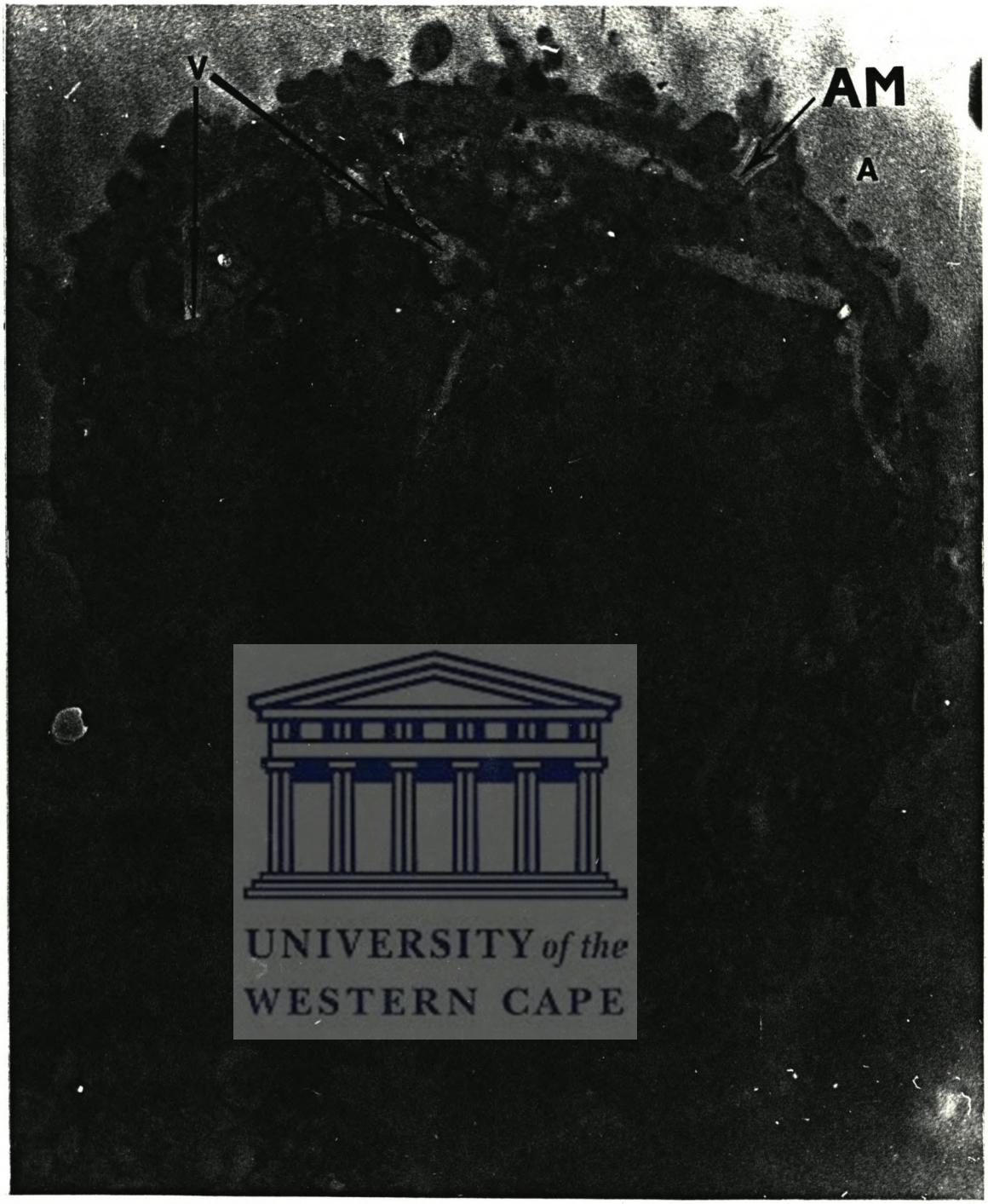


Fig 31. Transmission electron micrograph of an alveolar macrophage (AM) "foam cell" in the alveolar spaces (A) of BD9 rats treated with chlorphentermine at a dose of 40mg/kg body weight for six weeks. The foam cell contains many vacoules (V).

Magnification 6 000X

chlorphentermine only by the lack of a chlorine atom in the para-position, did not induce phospholipidosis[75]. The results found in our study is in general agreement with that found in the above mentioned studies.

5.3 ENUMERATION OF ALVEOLAR MACROPHAGES

Following the last dose of fenfluramine, chlorphentermine or saline the Wistar and BD9 rats were sacrificed and the lungs lavaged. The alveolar macrophages isolated from the treated and untreated rats were enumerated with a Coulter® counter. The results are summarised in tables 3 and 4. The Coulter® counter's aperture allows the reading of macrophage of sizes $\leq$ 100 μm. The typical alveolar macrophage has a diameter of 15-25 μm[79].

From these results, the percentage increase in macrophage counts of the treated rats, compared to the saline-treated rats was calculated (see tables 3 and 4). In the fenfluramine-treated Wistar and BD9 rats there were, compared to the saline-treated Wistar and BD9 rats, a 200 % and 235 % increase in macrophage counts, respectively (t-test; $p \leq 0.05$). In the chlorphentermine-treated Wistar and BD9 rats there were, compared to the saline-treated Wistar and BD9 rats, a 700 % and 965 % increase in macrophage counts, respectively. In the chlorphentermine-treated rats significantly higher (t-test; $p \leq 0.05$) macrophage counts was observed for Wistar rats (1.6×10^9) and BD9 rats (2.13×10^9) compared to that obtained for the fenfluramine-treated Wistar rats (0.6×10^9) and BD9 rats (0.67×10^9). This difference may be due to differences in the pharmacokinetics of fenfluramine and chlorphentermine, which may lead to differences in the extents of their accumulation in the lung[39] or it may indicate actual differences in the effects that each has on the macrophages of the two rat strains.

In general 85-90% of the free cells washed from the lungs of rabbits, rats and mice have characteristics which allow them to be identified as alveolar macrophages[80]. In guinea pigs, hamsters and monkeys it is reported that a lower percentage of cells removed are alveolar macrophages[81]. Differences in macrophage counts between species may be due to actual differences in the free cell population in the alveoli, damages associated with the lavage procedure which increases contamination from the capillaries, or undetected respiratory infection in the animals.

TABLE 3. MACROPHAGE COUNTS FOR LAVAGED LUNGS OF WISTAR RATS AFTER TREATMENT WITH CHLORPHENTERMINE, FENFLURAMINE AND SALINE.

TREATMENT	X 10^9 MACROPHAGES/LUNG ± SD (n = 4)
CHLORPHENTERMINE	1.6 ± 0.000 *
FENFLURAMINE	0.6 ± 0.100 *
SALINE	0.2 ± 0.058

TABLE 4. MACROPHAGES COUNTS FOR LAVAGED LUNGS OF BD9 RATS AFTER TREATMENT WITH CHLORPHENTERMINE, FENFLURAMINE AND SALINE.

TREATMENT	X 10^9 MACROPHAGES/LUNG ± SD (n = 4)
CHLORPHENTERMINE	2.13 ± 0.154 *u
FENFLURAMINE	0.67 ± 0.153 *
SALINE	0.2 ± 0.126

u * significantly different from value for saline treated animals. (t-test; $p \leq 0,05$)

One characteristic of the foam cell response is that the cells in the alveoli are quite variable in size[3]. By comparison, alveolar macrophages from normal animals are relatively uniform in size. Reasor et al[4] treated rats with chlorphentermine for 4 weeks and examined the phospholipid content of alveolar macrophages as a function of cell size. Using centrifugal elutriation, alveolar macrophages were separated into 4 subpopulations of increasing cell size. It was found that as the cells become larger, the total phospholipid content increases proportionally.

The subpopulation of the largest cells had a phospholipid content of 24 μmol phospholipid/10^7 cells compared to control alveolar macrophages which contained 0.4 μmol phospholipid/10^7 cells illustrating striking accumulation of phospholipids which occurs in some of the cells. It was therefore anticipated that the elevated number of hypertropied alveolar macrophages seen after the administration of fenfluramine or chlorphentermine in this study would also be accompanied by raised phospholipid levels in the lung.

5.4 PHOSPHOLIPID COMPOSITION OF UNLAVAGED AND LAVAGED LUNGS

Following the last dose, the Wistar and BD9 rats were sacrificed and the phospholipids extracted from the lavaged lungs of the fenfluramine, chlorphentermine- or saline-treated rats. The extracted phospholipids were separated by thin layer chromatography and quantified by means of a densitometer. The results are summarised in tables 5 to 8.

Both drugs caused an increase in pulmonary phospholipids[v] in the two species of rats. In the fenfluramine-treated Wistar and BD9 rats there were a 198.95 % and 38.79 % increase in total phospholipids (see tables 5 and 6) respectively (compared to the saline-treated Wistar and BD9 control rats), while chlorphentermine induced a 168.25 % and 86.28 % increase in total phospholipids in the Wistar and BD9 rats (see tables 5 and 6), respectively (compared to the saline-treated Wistar and BD9 control rats).

In both instances the Wistar rat appeared to be more sensitive to the drug-induced effect. Fenfluramine compared to chlorphentermine however caused a 30.70 % greater increase in total lung phospholipid levels in the Wistar rats while in the BD9 rats the situation was reversed with chlorphentermine, causing a 47.49 % greater increase in total lung phospholipid levels. These data prove that differences in the induction of individual phospholipids in the different strains may occur. We used two strains of rats in this investigation viz. Wistar and BD9, to account for the possibility of the drugs being more or less effective at inducing phospholipidosis because of the rat strain used. The data obtained confirmed that this had been a wise decision.

The ability of chlorphentermine to induce an accumulation of phospholipids principally in the alveolar macrophages and unlavaged lung is well documented[3]. The extent of the cellular accumulation of phospholipids after

---

[v] PC = Phosphatidyl choline, PE = Phosphatidyl ethanolamine, PI = Phosphatidyl inositol, PS = Phosphatidyl serine, PG = Phosphatidyl glycerol, SPM = Sphingomyelin and PA = Phosphatidic acid.

chlorphentermine administration is dependent on the duration of drug treatment and the dose at which it is administered[82]. Seiler et al[34] found that chronic daily administration of approximately 50 mg/kg chlorphentermine for 4 to 8 weeks significantly elevates the levels of PC, SPM and PE in the unlavaged lung. The results found in these quoted studies are consistent with what was found in the present study for chlorphentermine.

Lavaging of the lungs considerably reduced the levels of phospholipids remaining in the lung and the differences between the treated and untreated animals became less striking (tables 7 and 8). The total phospholipid levels found in the Wistar and BD9 rats after fenfluramine treatment were now found to be elevated by 84.73 % and 4.19 % (tables 7 and 8) respectively, and the corresponding values for the chlorphentermine-treated rats were 36.21 % and 31.62 % (tables 7 and 8) higher respectively, compared to the saline-treated Wistar and BD9 control rats. Collectively these data confirm that most of the accumulated phospholipids were located in the alveolar spaces in both Wistar and BD9 rats.

After an extensive literature search no data was found to indicate which phospholipids may accumulate in the rat lung after treatment with fenfluramine. If we consider the fact that there are differences in the phospholipid levels induced by the different cationic amphiphilic drugs and that such differences may arise due to differences in the mechanisms by which these drugs cause phospholipids to accumulate, a major objective of this thesis was to analyse the levels and type of phospholipids induced by fenfluramine. Such information may give us a clearer insight into the mechanism of this drug-induced phenomenon.

TABLE 5: PHOSPHOLIPID LEVELS (mg/g DRY LUNG ± SD) IN UNLAVAGED LUNGS OF WISTAR RATS TREATED WITH CHLORPHENTERMINE, FENFLURAMINE OR SALINE FOR SIX WEEKS

PHOSPHOLIPID	CHLORPHENTERMINE n = 4	FENFLURAMINE n = 4	SALINE n = 4
PC %A[w] %B[x]	30.12 ± 1.20 *[y] 62.1 +216	28.95 ± 3.87 * 53.6 +204	9.53 ± 1.07 52.7
PE %A %B	1.17 ± 0.05 * 2.4 +15	2.75 ± 0.15 * 5.1 +170	1.02 ± 0.04 5.6
PG %A %B	0.61 ± 0.02 * 1.3 -46	1.12 ± 0.02 2.1 -2	1.14 ± 0.21 6.3
PI %A %B	0.47 ± 0.01 * 1 +104	0.87 ± 0.02 * 1.6 +278	0.23 ± 0.00 1.3
PS %A %B	0.21 ± 0.25 0.4 -16	0.49 ± 0.94 * 0.9 +96	0.25 ± 0.29 1.38
SPM %A %B	15.06 ± 1.05 * 31 +174	19.37 ± 1.40 * 36 +252	5.50 ± 0.27 30
PA %A %B	0.86 ± 0.02 * 1.8 +110	0.50 ± 0.03 * 0.9 +22	0.41 ± 0.00 2.3
TOTAL PHOSPHOLIPID %A %B	48.50 100 +168	54.05 100 +199	18.08 100

[w] $$\% \text{ of total phospholipid} = \frac{[\text{phospholipid}] \text{ after treatment}}{[\text{total phospholipid}] \text{ after treatment}} \times 100$$

[x] % increase compared to saline level =

$$\frac{[\text{phospholipid}] \text{ after treatment} - [\text{phospholipid}] \text{ after saline}}{[\text{phospholipid}] \text{ after saline}} \times 100$$

[y] * significantly different from value for saline treated animals (t-test ; $p \leq 0,05$)

TABLE 6: PHOSPHOLIPID LEVELS (mg/g DRY LUNG ± SD) IN UNLAVAGED LUNGS OF BD9 RATS TREATED WITH CHLORPHENTERMINE, FENFLURAMINE OR SALINE FOR SIX WEEKS.

PHOSPHOLIPID	CHLORPHENTERMINE n = 4	FENFLURAMINE n = 4	SALINE n = 4
PC %A[z] %B[aa]	47.64 ± 1.11 * [bb] 62 +106	30.48 ± 3.99 * 53.6 +32	23.18 ± 1.85 56.6
PE %A %B	1.39 ± 0.20 * 1.8 +9	1.62 ± 0.05 * 2.9 +26	1.28 ± 0.04 3.1
PG %A %B	0.34 ± 0.04 0.4 -23	0.34 ± 0.04 0.6 -23	0.44 ± 0.20 1.1
PI %A %B	0.78 ± 0.03 * 1.0 +63	0.69 ± 0.02 * 1.2 +44	0.48 ± 0.01 1.2
PS %A %B	0.18 ± 0.16 0.24 -14	0.26 ± 0.02 0.5 +24	0.21 ± 0.11 0.5
SPM %A %B	25.85 ± 0.81 * 33.9 +71	23.29 ± 0.85 * 41 +54	15.16 ± 1.10 37
PA %A %B	0.12 ± 0.01 * 0.16 -43	0.17 ± 0.02 * 0.3 -19	0.21 ± 0.00 0.5
TOTAL PHOSPHOLIPID %A %B	6.3 100 +86	56.85 100 +39	40.96 100

[z] $$\% \text{ of total phospholipid} = \frac{\text{[phospholipid] after treatment}}{\text{[total phospholipid] after treatment}} \times 100$$

[aa] % increase compared to saline level =

$$\frac{\text{[phospholipid] after treatment - [phospholipid] after saline}}{\text{[phospholipid] after saline}} \times 100$$

[bb] * significantly different from value for saline treated animals (t-test ; $p \leq 0,05$)

TABLE 7: PHOSPHOLIPID LEVELS (mg/g DRY LUNG ± SD) IN LAVAGED LUNGS OF WISTAR RATS TREATED WITH CHLORPHENTERMINE, FENFLURAMINE OR SALINE FOR SIX WEEKS.

PHOSPHOLIPID	CHLORPHENTERMINE n = 4	FENFLURAMINE n = 4	SALINE n = 4
PC %A[cc] %B[dd]	10.51 ± 2.25 40 +28	16.29 ± 3.05 *[cc] 47 +98	8.21 ± 3.20 44
PE %A %B	0.81 ± 0.13 3.2 +16	0.69 ± 0.17 2 -1.4	0.70 ± 0.15 3.7
PG %A %B	0.08 ± 0.02 * 0.3 78	0.14 ± 0.00 * 0.4 -62	0.37 ± 0.01 1.96
PI %A %B	0.49 ± 0.05 * 2 +81	0.43 ± 0.07* 1.2 +59	0.27 ± 0.04 1.4
PS %A %B	0.25 ± 0.07 0.9 +25	0.21 ± 0.15 0.6 +5	0.20 ± 0.04 1.0
SPM %A %B	13.12 ± 1.24 * 51 +46	16.83 ± 1.24 * 48 +87.4	8.98 ± 1.81 47.6
PA %A %B	0.43 ± 0.05 * 1.7 +230	0.25 ± 0.03 * 0.7 +108	0.13 ± 0.01 0.7
TOTAL PHOSPHOLIPID %A %B	25.69 100 +36	34.84 100 +84	18.86 100

[cc] % of total phospholipid = $\frac{\text{[phospholipid] after treatment}}{\text{[total phospholipid] after treatment}} \times 100$

[dd] % increase compared to saline level = $\frac{\text{[phospholipid] after treatment - [phospholipid] after saline}}{\text{[phospholipid] after saline}} \times 100$

[cc] * significantly different from value for saline treated animals (t-test ; $p \leq 0,05$)

TABLE 8: PHOSPHOLIPID LEVELS (mg/g DRY LUNG ± SD) IN LAVAGED LUNGS OF BD9 RATS TREATED WITH CHLORPHENTERMINE, FENFLURAMINE OR SALINE

PHOSPHOLIPID	CHLORPHENTERMINE n = 4	FENFLURAMINE n = 4	SALINE n = 4
PC %A[ff] %B[gg]	8.39 ± 2.42 35.4 +39	5.09 ± 1.38 27.3 -16	6.03 ± 1.52 33.7
PE %A %B	1.04 ± 0.10 4 +14	0.83 ± 0.10 4 -8.8	0.91 ± 0.06 5
PG %A %B	0.35 ± 0.02 * 1.5 -5.7	0.25 ± 0.02 *[hh] 1.3 0	0.43 ± 0.02 2.4
PI %A %B	0.33 ± 0.11 1.4 -43	0.35 ± 0.02 1.9 0	0.35 ± 0.02 1.95
PS %A %B	0.12 ± 0.02 * 0.5 -43	0.21 ± 0.28 1.1 0	0.21 ± 0.03 1.1
SPM %A %B	13.31 ± 2.07* 56 +35.7	11.79 ± 1.81 * 63 +20	9.81 ± 1.90 55
PA %A %B	0.18 ± 0.01 * 0.75 +12.5	0.13 ± 0.00 * 0.7 -19	0.16 ± 0.01 0.89
TOTAL PHOSPHOLIPID %A %B	23.72 100 +31.6	18.65 100 +4.2	17.90

[ff] % of total phospholipid = $\frac{\text{[phospholipid] after treatment}}{\text{[total phospholipid] after treatment}} \times 100$

[gg] % increase compared to saline level =

$$\frac{\text{[phospholipid] after treatment - [phospholipid] after saline}}{\text{[phospholipid] after saline}} \times 100$$

[hh] * significantly different from value for saline treated animals (t-test ; $p \leq 0.05$)

Indeed, at the dosage used in this study, definite trends appear to have emerged with respect to the accumulated phospholipids (see tables 5 to 8). In the unlavaged lungs from the Wistar rats, significant increases occured in all phospholipids after treatment with both drugs, except PG and PS in the chlorphentermine-treated rats and PG in the fenfluramine-treated rats. This may suggest that there is a subtle difference in the mechanism of phospholipid accumulation induced with fenfluramine and chlorphentermine in this rat specie. This however must be seen against the background fact that PS and PG only constituted 1.7 % of the total phospholipid for chlorphentermine-treated Wistar rats vs 3.0 % for the fenfluramine-treated Wistar rats. It therefore appears that in the Wistar rats fenfluramine and chlorphentermine may cause the same phospholipids to accumulate in the lung and probably by similar mechanisms. With respect to the unlavaged lungs of the BD9 rats, the levels of all the phospholipids were found to be significantly higher after treatment with both drugs, except that of PG, PS and PA (compared to the saline-treated BD9 controls). This again may suggest that there is a subtle difference in the mechanism of phospholipid accumulation induced with fenfluramine and chlorphentermine in this species of rat but, if one, again considers the fact that these phospholipids (PS, PG and PA) together only constitute 1.4 % of the total phospholipid after fenfluramine treatment and 0.8 % of the total phospholipid after chlorphentermine treatment in the unlavaged rats (table 6), the evidence for a difference in the mechanism of phospholipid accumulation after fenfluramine or chlorphentermine treatment is however not too strong. It therefore appears that in both Wistar and BD9 rats fenfluramine and chlorphentermine cause the same phospholipids to accumulate in the lung and probably by similar mechanisms. Small quantitative differences in these similar mechanisms (e.g. differences in the degree of binding), rather than different mechanisms would, in my opinion, be the most likely to account for the small differences in elevated phospholipids obtained in this study.

The aetiology of drug-induced phospholipidosis may well be very complex with several mechanisms being involved. It would thus be of interest to ascertain whether the elevation of specific pulmonary phospholipids found in the present study, are due to (a) direct inhibition of phospholipases by chlorphentermine or fenfluramine or (b) the result of inhibition of the phospholipases by the specific drug-phospholipid complexes.

5.5 EFFECTS OF CHLORPHENTERMINE AND FENFLURAMINE ON PHOSPHOLIPASE ACTIVITY

The results of the studies of the effect of chlorphentermine and fenfluramine on phospholipase-A- and -C-mediated hydrolyses of all the phospholipids[ii] are depicted in tables 9 to 22.

(a) PHOSPHOLIPASE-A ACTIVITY

All the phospholipids were substrates for determining the phospholipase-A (PL-A) activity. Values for PL-A mediated- hydrolyses ranged from a minimum of 0.811 to a maximum of 4.04 μg FFA μl^{-1} sample min^{-1}, for the range of substrates used.

As suspected might happen, the addition of chlorphentermine or fenfluramine whether directly to the enzyme or in association with the phospholipid resulted in significant decreases in the PL-A mediated hydrolyses of virtually all the phospholipids. To ascertain whether there is a preference for one of these two modes of inhibition eg. direct inhibition of the enzyme or phospholipid substrate-drug complex-mediated inhibition to prevail, we compared the % inhibition obtained for the PL-A-chlorphentermine complex

[ii] PL = phospholipid

vs that for the phospholipid substrate-chlorphentermine complex. In the case of the PL-A-drug complexes a small but significantly greater degree of inhibition of the hydrolysis of all the phospholipids except PI and PA (see tables 9 to 22) compared to that for the corresponding phospholipid-drug complex was obtained. Inturn for PI- and PA, the phospholipid-drug complexes induced a significantly greater inhibition compared to that for the PL-A-drug complexes. Although statistically different, the above differences were (apart from the PI and PA) however not of such a magnitude to serve as conclusive proof that the one mode of inhibition is more likely to prevail than the other.

For fenfluramine, in the case of phospholipids PI, PS and PA, the % inhibition via the phospholipid substrate-drug complex mechanism was significantly higher than that for the PL-A-drug complex mechanism (see tables 12, 13 and 15). The PL-A-drug complexes inturn showed a significantly greater inhibition of the hydrolysis of the PC and SPM (see table 9 and 14). No differences were found between the PL-A-drug and phospholipid substrate-drug complexes for PE and PG. It would thus appear that the fenfluramine-induced accumulation of PI, PS and PA may be the result of phospholipid substrate modification. On the otherhand the accumulation of PC and SPM may be the result of a direct inhibition of PL-A activity. Although statistically different the above differences, for PC and SPM were however also not of such a magnitude to serve as conclusive proof that the one mode of inhibition is more likely than the other.

TABLE 9 PL-A ACTIVITY FOLLOWING THE ENZYMATIC BREAKDOWN OF PHOSPHATIDYL CHOLINE

SAMPLE	μg FFA[ii] μl^{-1} SAMPLE min^{-1} $\pm$ SD n = 3	% INHIBITION
PC CONTROL[kk]	4.77 $\pm$ 0.0001	
PC + CP COMPLEX[ll]	0.307 $\pm$ 0.0002 *[mm]	93.56
PC + FF COMPLEX	0.263 $\pm$ 0.0001 *	94.48
PC + CP ENZYME[nn]	0.204 $\pm$ 0.0003 *	95.72
PC + FF ENZYME	0.216 $\pm$ 0.0002 *	95.47

TABLE 10. PL-A ACTIVITY FOLLOWING THE ENZYMATIC BREAKDOWN OF PHOSPHATIDYL ETHANOLAMINE

SAMPLE	μg FFA μl^{-1} SAMPLE min^{-1} $\pm$ SD n = 3	% INHIBITION
PE CONTROL	1.89 $\pm$ 0.002	
PE + CP COMPLEX	0.21 $\pm$ 0.001 *	88.89
PE + FF COMPLEX	0.15 $\pm$ 0.000 *	92.06
PE + CP ENZYME	0.12 $\pm$ 0.000 *	93.65
PE + FF ENZYME	0.15 $\pm$ 0.001 *	92.06

[ii] FFA = free fatty acid

[kk] PL-CONTROL = phospholipid micelles vortexed with 300 μl water.

[ll] FF COMPLEX and CP COMPLEX = Drug substrate complex

[mm] * significantly different from value for saline treated animals (t-test ; $p \leq 0.05$)

[nn] FF ENZYME and CP ENZYME = Drug enzyme complex

TABLE 11. PL-A ACTIVITY FOLLOWING THE ENZYMATIC BREAKDOWN OF PHOSPHATIDYL GLYCEROL

SAMPLE	μg FFA μl^{-1} SAMPLE min^{-1} ± SD n = 3	% INHIBITION
PG CONTROL	4.04 ± 0.004	
PG + CP COMPLEX	0.34 ± 0.002 *∞	91.58
PG + FF COMPLEX	0.15 ± 0.001 *	96.29
PG + CP ENZYME	0.21 ± 0.002 *	94.80
PG + FF ENZYME	0.167 ± 0.002 *	95.86

TABLE 12. PL-A ACTIVITY FOLLOWING THE ENZYMATIC BREAKDOWN OF PHOSPHATIDYL INOSITOL

SAMPLE	μg FFA μl^{-1} SAMPLE min^{-1} ± SD n = 3	% INHIBITION
PI CONTROL	0.798 ± 0.0006	
PI + CP COMPLEX	0.202 ± 0.002 *	74.69
PI + FF COMPLEX	0.110 ± 0.0007 *	86.22
PI + CP ENZYME	0.453 ± 0.001	43.23
PI + FF ENZYME	0.223 ± 0.001 *	72.06

∞ * significantly different from value for saline treated animals (t-test ; $p \leq 0,05$)

TABLE 13: PL-A ACTIVITY FOLLOWING THE ENZYMATIC BREAKDOWN OF PHOSPHATIDYL SERINE

SAMPLE	μg FFA μl^{-1} SAMPLE min^{-1} ± SD n = 3	% INHIBITION
PS CONTROL	1.223 ± 0.0007	
PS + CP COMPLEX	0.17 ± 0.0005 *	86.10
PS + FF COMPLEX	0.11 ± 0.0004 *	91.01
PS + CP ENZYME	0.12 ± 0.001 *	90.18
PS + FF ENZYME	0.19 ± 0.0005 *	84.46

TABLE 14: PL-A ACTIVITY FOLLOWING THE ENZYMATIC BREAKDOWN OF SPHINGOMYELIN

SAMPLE	μg FFA μl^{-1} SAMPLE min^{-1} ± SD n = 3	% INHIBITION
SPM CONTROL	3.560 ± 0.009	
SPM + CP COMPLEX	0.215 ± 0.002 *	93.96
SPM + FF COMPLEX	0.127 ± 0.0006 *	95.78
SPM + CP ENZYME	0.171 ± 0.0004 *	95.20
SPM + FF ENZYME	0.15 ± 0.0007 *	96.43

TABLE 15: PL-A ACTIVITY FOLLOWING THE ENZYMATIC BREAKDOWN OF PHOSPHATIDIC ACID

SAMPLE	μg FFA μl^{-1} SAMPLE min^{-1} ± SD n = 3	% INHIBITION
PA CONTROL	0.811 ± 0.001	
PA + CP COMPLEX	0.22 ± 0.0007 *pp	72.87
PA + FF COMPLEX	0.18 ± 0.002 *	77.81
PA + CP ENZYME	0.30 ± 0.003 *	63.01
PA + FF ENZYME	0.391 ± 0.003 *	51.79

Phosphatidyl ethanolamine and PG appear to accumulate to a similar extent due to both direct inhibition of PL-A activity and inhibition via the phospholipid substrate-drug complex. From all the above considered collectively, it is thus not possible to declare either direct inhibition of the PL-A or phospholipid substrate modification as the more likely mechanism for either chlorphentermine or fenfluramine-induced inhibition of PL-A.

(b) PHOSPHOLIPASE-C ACTIVITY

All the phospholipids were found to be substrates for determining the phospholipase-C (PL-C) activity. Values for the PL-C mediated hydrolyses ranged from a minimum of 0.023 to a maximum of 0.827 μg IP 100 μl^{-1} sample min^{-1}, for the range of substrates used.

Again we analysed the data to ascertain whether there was a preference for one of the two modes of inhibition i.e. direct inhibition of the enzyme or phospholipid substrate-drug complex-mediated inhibition. For

pp * significantly different from value for saline treated animals (t- test; $p \leq 0,05$)

chlorphentermine the use of the phospholipid substrate-drug complexes resulted in a significantly greater inhibition for all phospholipids except SPM, compared to that for the PL-C-drug complexes (see tables 16 to 22). Inturn the PL-C-drug complex induced a significantly greater degree of inhibition of the hydrolysis of SPM (see table 21). It would thus appear that the chlorphentermine-induced accumulation of PC, PE, PG, PI, PS and PA may be the result of phospholipid substrate modification and SPM accumulation the result of a direct inhibition of PL-C.

In the case of fenfluramine, the phospholipid substrate-drug complexes again resulted in a significantly greater inhibition of the PL-C mediated hydrolyses of all phospholipids (except PE, PS and SPM), compared to the PL-C-drug complexes (see tables 16 to 22). The PL-C-drug complex inturn resulted in a significantly greater degree of inhibition of the hydrolyses of PE, PS and SPM (see tables 17, 20 and 21). It therefore appears that the fenfluramine-induced accumulation of PC, PG, PI and PA may be the result of phospholipid-substrate modification, and PE, PS and SPM due to the direct inhibition of PL-C activity.

Overall our data thus shows that both drugs have the ability to inhibit the PL-A activity by greater than 80 % by both direct complexation for all phospholipids (except in the case of PI and PA) and by the phospholipid-drug complexation mechanisms, again for all the phospholipids (except in the case of PA and PI-fenfluramine complex). On the otherhand the direct inhibition of PL-C, by both drugs, does not appear to have a significant influence on the inhibition of the hydrolyses of any of the phospholipids studied, except PE. Collectively, it is evident that our data supports the notion that both fenfluramine- and chlorphentermine-induced accumulation of phospholipids in the treated Wistar and BD9 rats (see section 5.4), most likely occur via similar mechanisms of inhibition of phospholipase activity.

TABLE 16: PL-C ACTIVITY FOLLOWING THE ENZYMATIC BREAKDOWN OF PHOSPHATIDYL CHOLINE

SAMPLE	μg IP[qq] 100μl^{-1} SAMPLE min^{-1} ± SD n = 3	% INHIBITION
PC CONTROL[rr]	0.342 ± 0.010	
PC + CP COMPLEX	0.026 ± 0.004 *[ss]	92.40
PC + FF COMPLEX	0.085 ± 0.005 *	75.15
PC + CP ENZYME	0.319 ± 0.010	6.73
PC + FF ENZYME	0.314 ± 0.011	8.18

TABLE 17: PL-C ACTIVITY FOLLOWING THE ENZYMATIC BREAKDOWN OF PHOSPHATIDYL ETHANOLAMINE

SAMPLE	μg IP 100μl^{-1} SAMPLE min^{-1} ± SD n = 3	% INHIBITION
PE CONTROL	0.391 ± 0.006	
PE + CP COMPLEX	0.042 ± 0.001 *	89.26
PE + FF COMPLEX	0.046 ± 0,002 *	88.24
PE + CP ENZYME	0.059 ± 0.001 *	84.91
PE + FF ENZYME	0.040 ± 0.002 *	89.77

[qq] IP = inorganic phosphate

[rr] PL-CONTROL = phospholipid micelles vortexed with 300 μl water.

[ss] * significantly different from value for saline treated animals (t-test ; $p \leq 0,05$)

TABLE 18: PL-C ACTIVITY FOLLOWING THE ENZYMATIC BREAKDOWN OF PHOSPHATIDYL GLYCEROL

SAMPLE	μg IP 100μl^{-1} SAMPLE min^{-1} ± SD n = 3	% INHIBITION
PG CONTROL	0.451 ± 0.021	
PG + CP COMPLEX	0.154 ± 0.005 *	65.85
PG + FF COMPLEX	0.127 ± 0.001*	71.84
PG + CP ENZYME	0.397 ± 0.024	11.97
PG + FF ENZYME	0.383 ± 0.012	15.10

TABLE 19: PL-C ACTIVITY FOLLOWING THE ENZYMATIC BREAKDOWN OF PHOSPHATIDYL INOSITOL

SAMPLE	μg IP 100μl^{-1} SAMPLE min^{-1} ± SD n = 3	% INHIBITION
PI CONTROL	0.135 ± 0.004	
PI + CP COMPLEX	0.076 ± 0.004	43.70
PI + FF COMPLEX	0.023 ± 0.023 *	82.96
PI + CP ENZYME	0.077 ± 0.003	42.96
PI + FF ENZYME	0.094 ± 0.005	30.37

TABLE 20: PL-C ACTIVITY FOLLOWING THE ENZYMATIC BREAKDOWN OF PHOSPHATIDYL SERINE

SAMPLE	μg IP $100\mu l^{-1}$ SAMPLE min^{-1} $\pm$ SD n = 3	% INHIBITION
PS CONTROL	0.342 $\pm$ 0.006	
PS + CP COMPLEX	0.023 $\pm$ 0.005 *tt	93.27
PS + FF COMPLEX	0.197 $\pm$ 0.006	42.39
PS + CP ENZYME	0.147 $\pm$ 0.007 *	57.02
PS + FF ENZYME	0.185 $\pm$ 0.011	45.91

TABLE 21: PL-C ACTIVITY FOLLOWING THE ENZYMATIC BREAKDOWN OF SPHINGOMYELIN

SAMPLE	μg IP $100\mu l^{-1}$ SAMPLE min^{-1} $\pm$ SD n = 3	% INHIBITION
SPM CONTROL	0.367 $\pm$ 0.003	
SPM + CP COMPLEX	0.264 $\pm$ 0.006	28.07
SPM + FF COMPLEX	0.349 $\pm$ 0.002	4.9
SPM + CP ENZYME	0.212 $\pm$ 0.016	42.23
SPM + FF ENZYME	0.219 $\pm$ 0.001	40.33

tt * significantly different from value for saline treated animals (t-test ; $p \leq 0.05$)

TABLE 22: PL-C ACTIVITY FOLLOWING THE ENZYMATIC BREAKDOWN OF PHOSPHATIDIC ACID

SAMPLE	μg IP $100\mu l^{-1}$SAMPLEmin^{-1} ± SD n = 3	% INHIBITION
PA CONTROL	0.827 ± 0.010	
PA + CP COMPLEX	0.479 ±0.012*[uu]	42.08
PA + FF COMPLEX	0.480 ± 0.030*	41.96
PA + CP ENZYME	0.787 ± 0.023	4.84
PA + FF ENZYME	0.794 ± 0.012	3.90

Studies done by Lüllman et al[1] showed that cationic amphiphilic drugs (of which fenfluramine and chlorphentermine are examples) bind to phospholipids and that the resulting complexes are resistant to phospholipase action. Exactly how fenfluramine or chlorphentermine becomes bound to alveolar phospholipids is not known but Seydel and Wasserman[83] with the aid of NMR[vv] studies provided evidence of such drug-phospholipid binding. To investigate further this most likely mechanism for the elevated lung phospholipids levels induced by fenfluramine and chlorphentermine viz. the one of complex formation between the drug and the phospholipid, we monitored the effect of these drugs on the phase transition temperature of the phospholipids. It is reported that the amount of depression of the phase transition temperature, is dependent on the degree of binding of the drug to the phospholipid.

[uu] * significantly different from value for saline treated animals (t-test ; $p \leq 0,05$)

[vv] NMR = Nuclear magnetic resonance

5.6 EFFECTS OF CHLORPHENTERMINE AND FENFLURAMINE ON THE PHASE TRANSITION TEMPERATURE OF PHOSPHOLIPIDS

The results of studies of the effect of chlorphentermine and fenfluramine on the phase transition temperature of all the phospholipids are shown in tables 23 to 29. The phase transition temperatures for the phospholipid controls ranged from a minimum of 34.81 °C to a maximum of 94.65 °C (see tables 23 to 29). After an extensive literature search only the values for the phase transition temperatures of PC, PG and PA were obtained, and these were 41, 42 and 63 °C respectively[84]. Our data obtained for the phospholipid controls PC, PG and PA (see tables 23, 25 and 29) do not correspond to these values reported in the literature. A possible explanation for this discrepancy between the reported data and our controls, may be the difference in the method of phospholipid substrate preparation. We used Triton X-100 for the preparation of the mixed micelles, since this was used in the preparation of the mixed micelles in the PL-A and PL-C enzyme studies, while in the literature-reported studies phospholipid liposomes were prepared without the use of Triton X-100. After an extensive literature search no phase transition studies could in fact be found where Triton X-100 was used in the preparation of the liposomes.

In general the phase transition temperature varies with the charge carried by the phospholipid head groups. Phospholipids with negatively charged headgroups favour a fluid phase, leading to a lower phase transition temperature than that of an uncharged, but otherwise similar phospholipid. The bulky head group of PC confers unto this phospholipid a lower phase transition temperature than it would, to a similar phospholipid containing a smaller head group. For example the phase

transition temperature of PC is 41 °C while that of PE is 63 °C[85]. How Triton X-100 would influence the above considerations is not entirely clear but, even if the inclusion of Triton X-100 affected the phase transition of the phospholipids (through one or more of the above considerations), it can still be argued that the effect of each of the drugs i.e. chlorphentermine or fenfluramine on the phase transition temperature, if produced in a similar way, would most likely be similarly affected and thus the inclusion of the Triton X-100 should not affect the conclusions drawn in these studies.

The phase transition temperatures for all the chlorphentermine-phospholipid complexes showed a significant downward shift compared to the phospholipid controls (see tables 23 to 29). This is in agreement with studies done by Mohr et al[73,86] who showed that chlorphentermine caused a depression of the phase transition temperature for phospholipids compared to the controls. They postulated that the extent to which the phase transition temperature is depressed by a drug may depend on the concentration of drug within the phospholipid micelle. The location of the charged cationic-amphiphilic drug molecule (see figure 1) will be governed by the electrostatic attraction between the negatively charged phosphate moiety of the phospholipid and the positively charged nitrogen atom of the drug[87]. In the second phase of the interaction hydrophobic bonds might cause an association of the aromatic ring of the drug to the hydrophobic moiety of the phospholipid molecule (or phospholipid micelle). The tendency to form an association of this kind can be expected to be enhanced with increasing apolarity (hydrophobicity) of the ring structure[87].

TABLE 23. PHASE TRANSITION TEMPERATURE OF PHOSPHATIDYL CHOLINE

SAMPLE	PTT[ww] ± SD n = 3	ΔT[xx]	ΔT%[yy]
PC CONTROL	34.81 ± 0.015	-	-
PC+FF COMPLEX	24.32 ± 0.266	10.49	30.14
PC+CP COMPLEX	29.08 ± 0.021	5.73	16.46

TABLE 24. PHASE TRANSITION TEMPERATURE OF PHOSPHATIDYL ETHANOLAMINE

SAMPLE	PTT ± SD n = 3	ΔT	ΔT%
PE CONTROL	88.08 ± 0.006	-	-
PE+FF COMPLEX	73.85 ± 0.006	14.23	16.16
PE+CP COMPLEX	76.67 ± 0.026	11.41	12.95

TABLE 25: PHASE TRANSITION TEMPERATURE OF PHOSPHATIDYL GLYCEROL

SAMPLE	PTT ± SD n = 3	ΔT	ΔT%
PG CONTROL	74.15 ± 0.006	-	-
PG+FF COMPLEX	70.44 ± 0.037	3.71	5
PG+CP COMPLEX	63.10 ± 0.006	11.05	14.9

[ww] PTT = PHASE TRANSITION TEMPERATURE (°C)

[xx] ΔT = The difference in the transition temperature between the phospholipid control and the phospholipid-drug complex. (° C)

[yy] ΔT% = The relative change in the phase transition temperature.

TABLE 26: PHASE TRANSITION TEMPERATURE OF PHOSPHATIDYL INOSITOL

SAMPLE	PTT[zz] ± SD n = 3	ΔT[aaa]	ΔT%
PI CONTROL	90.47 ± 0.010	-	-
PI+FF COMPLEX	83.24 ± 0.010	7.23	7.99
PI+CP COMPLEX	85.19 ± 0.005	5.28	5.84

TABLE 27: PHASE TRANSITION TEMPERATURE OF PHOSPHATIDYL SERINE

SAMPLE	PTT ± SD n = 3	ΔT	ΔT%
PS CONTROL	94.65 ± 0.049	-	-
PS+FF COMPLEX	88.90 ± 0.006	5.75	6.08
PS+CP COMPLEX	78.92 ± 0.020	15.73	16.62

TABLE 28: PHASE TRANSITION TEMPERATURE OF SPHINGOMYELIN

SAMPLE	PTT ± SD n = 3	ΔT	ΔT%
SPM CONTROL	38.37 ± 0.017	-	-
SPM+FF COMPLEX	37.85 ± 0.010	0.52	1.36
SPM+CP COMPLEX	30.38 ± 0.011	7.99	20.82

[zz] PTT = PHASE TRANSITION TEMPERATURE (°C)

[aaa] ΔT = The difference in the transition temperature between the phospholipid control and the phospholipid-drug complex. (°C).

TABLE 29: PHASE TRANSITION TEMPERATURE OF PHOSPHATIDIC ACID

SAMPLE	PTT ± SD n = 3	ΔT	ΔT%
PA CONTROL	92.56 ± 0.015	-	-
PA+FF COMPLEX	86.11 ± 0.005	6.45	6.97
PA+CP COMPLEX	84.45 ± 0.006	8.11	8.76

All phospholipids have a negative charge at the phosphate group at pH 7; the pKa of the phospholipids is in the range of 1 to 2. One has to distinguish between the binding of drugs to zwitterionic polar lipids (PC, PE and SPM) not possesing a nett electric charge, and to polar lipids with a negative nett electric charge (PI, PG, PS and PA) see figure 32. The zwitterionic structure provides strong electrostatic attraction between hydrophilic head groups, maintaining a close knit structure[87]. Ionisation of the amine base depends on the hydrogen ion concentration and the pKa of the base; for most basic drugs the pKa falls in the range of 8 and above[87]. An extension of this principle leads to the conclusion that cationic amphiphilic drugs will find their most favourable environment associated with anionic phospholipids. The positive charge of the drug located in the region of the negative head groups will diminish the repulsion between the polar moieties of the lipid molecules and thus reduce the space for intercalation. Such an effect has already been demonstrated for inorganic cations[28].

At the drug/phospholipid molar ratios used in the present experiment ie. for PC=1.98, PG=2.00 and PA=1.75 the literature reports phase

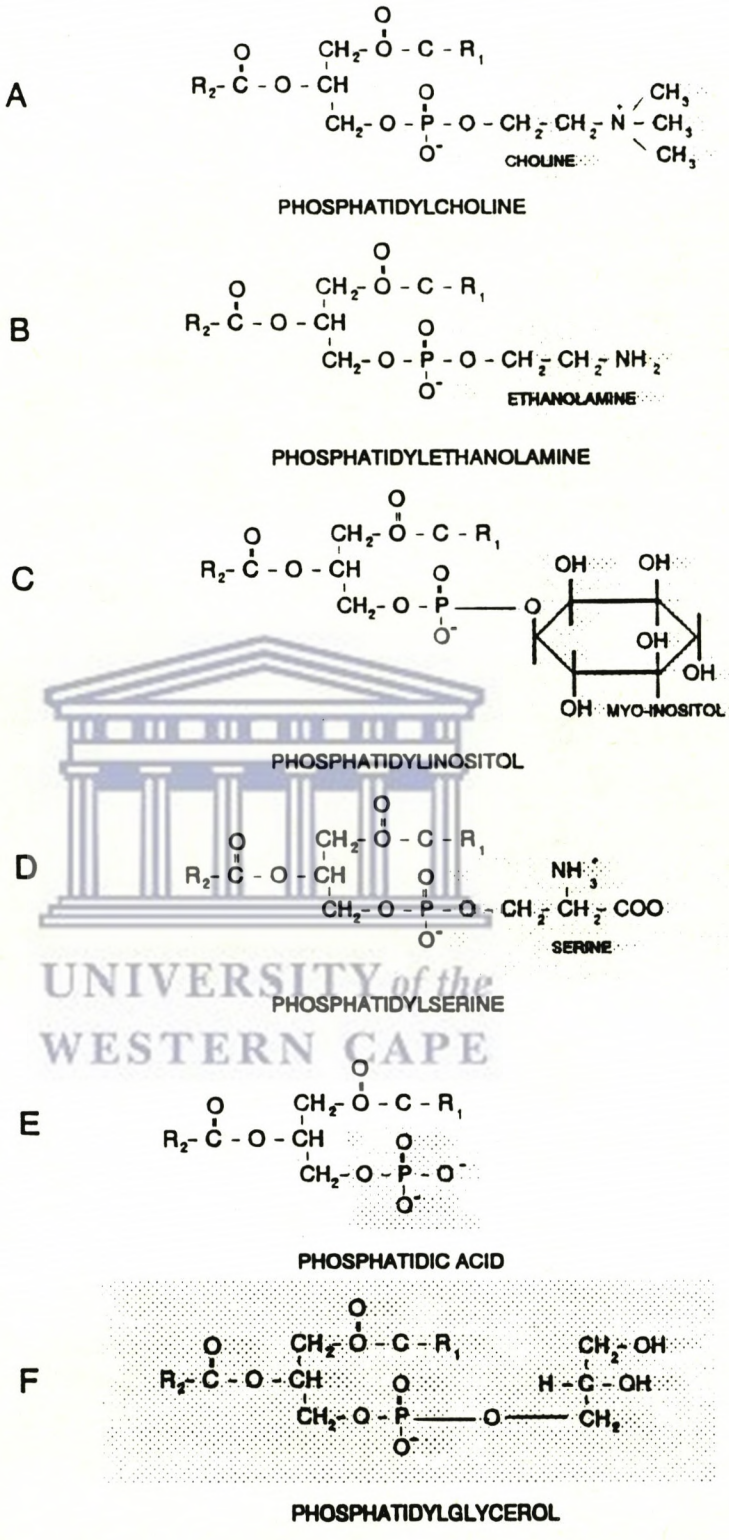


FIG. 32 Structures of phospholipids

transition temperatures of 32, 31 and 29 ° C for chlorphentermine-phospholipid complexes, respectively resulting in ΔT of 9, 11 and 34° C respectively[87]. Although our data for PG agreed with this, it did not for the PC and PA, respectively (see tables 23, 25 and 29). Based on the literature reports we expected a greater ΔT for the polar phospholipids (PC, PE and SPM), since the zwitterionic structure provides strong electrostatic attraction between hydrophilic head groups.

From our data we individually observed a higher ΔT for PC, PE, SPM, PG and PS compared to PI and PA which are in agreement with our expectation. Literature reports indicate that PG, although not a zwitterionic phospholipid, after complex formation with chlorphentermine causes a phase transition depression quantitatively similar to that of PC[86]. PS on the otherhand is a phospholipid with a nett negative charge at pH 7 but nevertheless binds strongly to chlorphentermine. The relative phase transition temperature depressions observed for all the individual phospholipid substrate-chlorphentermine complexes, are thus in agreement with those expected for these phospholipids.

We also wanted to compare the effects of fenfluramine on the phase transition temperature of the phospholipid-drug complex to that of chlorphentermine. Similar to the case of the chlorphentermine-phospholipid complexes, the phase transition temperature for the fenfluramine-phospholipid complexes all showed a significant downward shift compared to the phospholipid controls (see tables 23 to 29). After an extensive literature search no data was found to indicate the phase

transition temperatures after fenfluramine complex formation with individual phospholipids. In the absence of literature values and studies for fenfluramine, one would have to compare the fenfluramine results with that of chlorphentermine. We observed a higher ΔT for PC and PE (see tables 23 and 24), compared to PI and PA (see tables 26 and 29), which is in agreement with our expectations, based on literature reports for chlorphentermine. This observed behaviour of the SPM-, PG- and PS-fenfluramine complexes may be related to the greater polarity of the fenfluramine molecule. The relative phase transition temperature depression for the fenfluramine-PC, PE, PI and PA complexes observed is in agreement with those expected for these phospholipids, but not for SPM, PG and PS.

Chlorphentermine, compared to fenfluramine, caused a significantly greater downward shift of the phase transition temperature for all phospholipids except PC, PE and PI (see tables 25 to 29). From our data it would appear that chlorphentermine binds more effectively than fenfluramine to these phospholipids. On the other hand fenfluramine caused a significantly greater downward shift of the phase transition temperature for phospholipids PC, PE and PI compared to chlorphentermine. It would thus appear that fenfluramine might bind more effectively than chlorphentermine to these particular phospholipids. When comparing the effects of chlorphentermine with that of fenfluramine, we indeed found that chlorphentermine caused a greater depression of the phase transition temperature for PG, PS, SPM and PA. Fenfluramine, compared to chlorphentermine, inturn caused a greater

depression of the phase transition temperature for PC, PE and PI.

The differences between fenfluramine and chlorphentermine with respect to the depression of the phase transition temperature of the individual phospholipids may indicate differences in the binding capacities of these drugs. Studies done by Kursch et al[87] showed that the introduction of a chlorine atom into the aromatic ring enhanced the binding capacity of chlorphentermine compared to phentermine to PC. This finding suggest that electrostatic forces may also determine binding capacities. Slight differences in electrostatic or hydrophobic attraction may result in subtle differences in the binding of fenfluramine or chlorphentermine to the phospholipid. The hydrophobic moiety of fenfluramine (see figure 4), contains a trifluoromethyl group in the the m-position, which may be less apolar than the corresponding moiety of chlorphentermine, namely a chlorine atom, located in the *p*-position. This is due to the greater electronegativity of the flourine atom compared to the chlorine atom on the aromatic ring structure. The secondary amine of fenfluramine is also less polar i.e. more hydrophobic than the corresponding primary amine of chlorphentermine. Thus the amphiphilic character (i.e. hydrophilic and hydrophobic nature) of the fenfluramine molecule will be less pronounced than that of chlorphentermine[45]. These subtle differences may inturn be responsible for the differences in magnitude of the changes in the phase transition temperature observed with the two drugs. A more conclusive correlation between the differences in the effects that the two drugs (chlorphentermine and fenfluramine) had on the depression of the phase transition temperature of the individual phospholipids is however not

easily discernible from the present data. Nevertheless our results appear to support the notion that both chlorphentermine and fenfluramine complex with individual phospholipids, and in this way might prevent the phospholipids from being metabolised by phospholipases.

5.6.1 CORRELATION OF THE EFFECTS OF CHLORPHENTERMINE AND FENFLURAMINE ON THE PHASE TRANSITION TEMPERATURE, PHOSPHOLIPASE INHIBITION AND LUNG PHOSPHOLIPID LEVELS.

The express aim of this study was to establish whether the accumulation of phospholipids within the lung lysosomes, induced by chlorphentermine and fenfluramine is due to direct inhibition of PL-A or PL-C or by increased binding of the drug to the phospholipid. We further specifically investigated the differences between the two drugs with respect to the drug-phospholipid binding mechanism (of inhibition of phospholipase-induced hydrolysis) by observing the phase transition temperatures for the phospholipid substrate-drug complexes for each drug. It would be expected that differences in the degree of inhibition of the phospholipases by the phospholipid substrate-drug complexes, for individual phospholipids, might be reflected in differences in the degree of binding of each drug, to the individual phospholipids and hence the degree to which each drug may lower the phase transition temperature of the particular phospholipid complex (see tables 30 and 31).

All the individual phospholipid substrate-chlorphentermine complexes, showed a greater than 70 % inhibition of PL-A induced phospholipid hydrolysis, whereas only the chlorphentermine-PC, -PE and -PS

complexes inhibited the PL-C activity by greater than 70 % (see table 31). These phospholipid substrate-drug complexes inturn, showed a downward shift in the phase transition temperature, compared to that of the controls. This appears to confirm the notion that these resulting phospholipid substrate-drug complexes have the ability to inhibit both PL-A and PL-C activity. Therefore these phospholipids should most likely be elevated in the lung. In the unlavaged chlorphentermine-treated Wistar and BD9 rats PC and PE are indeed elevated but not so for PS (see tables 5 and 6). The data obtained from the treated Wistar and BD9 rats for PC and PE thus appear to support the hypothesis that the extent of chlorphentermine binding to these phospholipids in the lung might cause them to be resistant to PL-A and PL-C. On the otherhand the chlorphentermine-PG, -PI, -SPM and -PA-complexes most likely inhibit the PL-A activity to a greater extent than it does PL-C activity (see table 31). These phospholipid substrate-drug complexes also, caused a downward shift in the phase transition temperature. Furthermore, in the unlavaged chlorphentermine-treated Wistar rats, PI, SPM and PA were all elevated respectively, (except PG) compared to the levels in the saline treated rats. In the unlavaged chlorphentermine-treated BD9 rats, PG again together with PA were not elevated. The data obtained from the treated Wistar and BD9 rats for PI, SPM and PA (except PA in the BD9 rats) thus appear to support the notion that the extent of chlorphentermine binding to these phospholipids in the lung might cause them to be resistant to PL-A. In general the full interpretation of the above results are however not easy due inpart to the confounding influence that the species differences might have had and the fact that a single drug concentration as opposed to concentration ranges were used in our study

and the differences in the phospholipid substrate affinities of the phospholipases thus not accomodated. Furthermore the use of pure phospholipase samples, instead of phospholipases isolated from the lungs of Wistar and BD9 rats may also have influenced our data. This latter approach regarding the phospholipases was decided on to minimise interference and complication of the procedure. A clear conclusive correlation between the drug-induced changes in lung phospholipid levels (where many factors play a role), the degree of inhibition of the phospholipase-mediated hydrolysis of activity by the individual phospholipids and the degree to which the phase transition temperature is lowered after complexing with chlorphentermine is thus not easily discernible.

Similar results were found in the case of fenfluramine. The phospholipid substrate- fenfluramine complexes showed a greater than 70 % inhibition of both PL-A- and PL-C-induced phospholipid hydrolyses (except PS, SPM and PA in the case of PL-C) of all the individual phospholipids (see table 31). This appears to confirm the notion that the above phospholipid substrate-drug binding complexes have the ability to inhibit both PL-A and PL-C activity. These phospholipid substrate-drug complexes inturn caused a downward shift in the phase transition temperature (see tables 23 to 26). Therefore these phospholipids should be elevated in the lung. In the unlavaged Wistar and BD9 rats, all the above phospholipids were indeed elevated (except PG). The data obtained from the treated Wistar and BD9 rats for PC, PE, and PI appears to confirm the notion that the extent of fenfluramine binding to these phospholipids in the lung might cause them to be resistant to PL-A and PL-C. The fenfluramine-PS, -

SPM, and -PA complexes, respectively showed a significantly greater inhibition of PL-A-induced phospholipid hydrolysis compared to inhibition for the PL-C induced phospholipid hydrolysis (see table 31). These phospholipid substrate-drug complexes inturn, caused a downward shift in the phase transition temperature (see tables 27 to 29) compared to the controls. Thus we can conclude that the fenfluramine-PS, -SPM and -PA complexes would most likely inhibit PL-A to a greater extent than they do PL-C. Therefore these phospholipids should most likely also be elevated in the lung. In the unlavaged fenfluramine-treated Wistar and BD9 rats the above phospholipids were all elevated except PA in the BD9 rats (see tables 5 and 6). The data obtained from the treated Wistar and BD9 rats for PS, SPM and PA (except PA in the BD9 rats) appear to support the notion that the extent of fenfluramine binding to these phospholipids in the lung might cause them to be resistant to PL-A. Similar to the case of the chlorphentermine-treated Wistar and BD9 rats, no clear conclusive correlation between the drug-induced changes in lung phospholipid levels, the degree of inhibition of the phospholipase activity of the individual phospholipids and the degree to which the phase transition temperature is lowered after complexing with fenfluramine is easily discernible.

Finally, besides the effects of chlorphentermine and fenfluramine on the individual phospholipid levels, be it via direct complexation with PL-A or PL-C, or due to substrate modification, the influence of these drugs on the ratios of PC/SPM and PG/PI levels may also be important. Changes in the PC/SPM and PG/PI ratios may indicate the possible occurence of respiratory distress syndrome or lung injury[88]. The PC/SPM ratio is

TABLE 30: COMPARISON OF THE PHASE TRANSITION TEMPERATURE AND PERCENTAGE INHIBITION OF PL-A AND PL-C AFTER COMPLEX FORMATION BETWEEN FENFLURAMINE AND VARIOUS PHOSPHOLIPIDS.

PHOSPHOLIPID	% INHIBITION OF PL-A	% INHIBITION OF PL-C	ΔT °C
PC	94.5	75.0	10.49
PE	92.1	88.0	14.23
PG	96.3	72.0	3.71
PI	86.0	83.0	7.23
PS	92.0	42.0	5.75
SPM	96.0	5.0	0.52
PA	77.0	42.0	6.45

TABLE 31: COMPARISON OF THE PHASE TRANSITION TEMPERATURE AND PERCENTAGE INHIBITION OF PL-A AND PL-C AFTER COMPLEX FORMATION BETWEEN CHLORPHENTERMINE AND VARIOUS PHOSPHOLIPIDS.

PHOSPHOLIPID	% INHIBITION OF PL-A	% INHIBITION OF PL-C	ΔT °C
PC	93.5	92.0	5.73
PE	88.8	89.0	11.41
PG	91.5	66.0	11.05
PI	75.5	43.0	5.28
PS	86.0	93.0	15.73
SPM	94.0	28.0	7.99
PA	72.0	42.0	8.11

less than one prior to about the 31 st week of pregnancy in humans, then rises to about 2 by the 34th week and 8 at 39 weeks[18]. This results from an increase in PC synthesis, rather than a decrease in SPM formation. The PG/PI ratio inturn has been shown to be a more accurate predictor of respiratory distress syndrome[12]. A decrease in the PG/PI ratio similar to that seen after exposure to 100% oxygen, is observed during incidences of impaired surfactant production in various species[89], and changes in the phospholipid composition. This indicates that in the ventilated primates, like in humans with respiratory failure, the PG/PI ratio can be used as early predictor of lung injury[88]. Thus when PG is present in detectable amounts in fetal lung, respiratory distress rarely occurs despite a low PC/SPM ratio. Literature reports also indicate that changes in the PG/PI ratios have been observed after treatment with N-nitroso-N-methylurethane, 4-aminopyrazolo-(3,4-d)-pyrimidine and bleomycin[90]. In the present study, decreased lung levels of PG and increased levels of PI was found for both fenfluramine- and chlorphentermine-treated Wistar and BD9 rats compared to the saline-treated rats. This resulted in a decrease in the PG/PI ratios following fenfluramine or chlorphentermine treatment compared to the saline. For Wistar rats the PG/PI ratios for saline, chlorphentermine and fenfluramine are 5, 1.30 and 1.29 vs 0.9, 0.4 and 0.5 for the BD9 rats. The data for the Wistar rats show a sharp decrease in the PG/PI ratio for the chlorphentermine- and fenfluramine-treated rats compared to the saline rats. The decrease in the PG/PI ratios for the BD9 rats is less striking for the chlorphentermine- and fenfluramine-treated rats compared to the saline. We can thus conclude that changes in the PG/PI ratios may be a sensitive index for these drug-induced effects.

Because of the confounding nature of these results, obtained when looking at the phospholipids collectively, we decided to repeat the analysis by looking at just one lung phospholipid viz. PC. Several literature reports exist on the influence of chlorphentermine on pulmonary levels of PC and on the interaction of chlorphentermine with PC. In the present study both drugs caused an increase in pulmonary PC in the two species of rats (see tables 5 and 6). Chlorphentermine compared to fenfluramine however caused a 12 % greater increase in pulmonary levels in the Wistar rats while in the BD9 rats it caused a 74 % greater increase in pulmonary levels. In both instances the Wistar rats appeared to be more sensitive at elevating PC levels. The data thus indicate that strain differences in the induction of individual phospholipids may occur.

As far as the effect of the chlorphentermine and fenfluramine on PL-A and PL-C activity was concerned, the chlorphentermine-PC complex showed a 93.5 % and 92.0 % inhibition of PL-A and PL-C activity respectively (see table 31). This appears to confirm the notion that the chlorphentermine-PC complex has the ability to inhibit both PL-A and PL-C activity. The fenfluramine-PC complex inturn displayed a 94.5 % and 75 % inhibition of PL-A and PL-C activity respectively (see table 30). Similar to the chlorphentermine-PC complex, the fenfluramine-PC complex also appears to have the ability to inhibit both PL-A and PL-C activity albeit at different rates. Therefore one could expect PC to be elevated in the lung as has been found (see tables 5 and 6). But does

fenfluramine differ from chlorphentermine in the way in which it inhibits the PL-A and PL-C hydrolysis of PC ?. Our data does not suggest so particularly as far as the one mechanism (i.e. that of phospholipid substrate-drug mediated inhibition) is concerned. Both the chlorphentermine-PC complexes and fenfluramine-PC complexes, inturn showed a significant downward shift in the phase transition temperature i.e. 29.08 and 24.32 °C compared to the control 34.81 °C (see table 23). With reference to the direct inhibition of PL-A or PL-C by chlorphentermine and fenfluramine the PL-A-chlorphentermine complex showed a 95.75 % inhibition of PL-A activity and the PL-C-chlorphentermine complex showed a 6.73 % inhibition of PL-C activity (see tables 9 and 16). This appears to support the notion that chlorphentermine has the ability to inhibit PL-A directly to a greater extent than PL-C. The PL-A-fenfluramine complex showed a 95.47 % inhibition of PL-A activity and the PL-C-fenfluramine complex a 8.18 % inhibition of PL-C activity (see tables 9 and 16). This also appears to support the notion that fenfluramine has the ability to inhibit PL-A directly to a greater extent than PL-C.

By just looking at PC (as opposed to many phospholipids), a more clear conclusive correlation can be seen. The correlation between the drug-induced changes in lung PC levels, the degree of inhibition of the phospholipase activity and the degree to which the phase transition temperature is lowered after complexing with chlorphentermine or

fenfluramine is more clearly evident. If any, only small quantitative differences is likely to exist between the effects of the two drugs.

Collectively the data obtained in this study strongly indicate that both fenfluramine and chlorphentermine administration to Wistar and BD9 rats are likely to cause phospholipids to accumulate in the lung by similar mechanisms. The etiology of drug-induced phospholipidosis may well be very complex with several mechanisms being involved. Subtle quantitative differences in these similar mechanisms e.g. differences in the degree of binding, rather than different mechanisms would, in my opinion, however most likely account for the differences in the elevated phospholipids obtained in this study.

CHAPTER SIX

CONCLUSION

CONCLUSION

The objective of this study was to investigate the mechanism of fenfluramine-induced phospholipidosis. It was proposed to do this by histological examination of the lungs, comparative analysis of the phospholipids that accumulate in the lungs of Wistar and BD9 rats after chronic treatment with fenfluramine or chlorphentermine and by investigating the mechanism of phospholipase inactivation. The primary hypothesis to be tested was that fenfluramine would induce the elevation of the same phospholipids as chlorphentermine, because like chlorphentermine, it also induces phospholipidosis by either directly inhibiting phospholipases or by binding to the individual phospholipid substrate, a process which renders the phospholipids less susceptible to hydrolysis by the phospholipases. The following major conclusions may be drawn from the results of this investigation:

(1) ***The phospholipidosis induced by fenfluramine in the lungs of both Wistar and BD9 rats are histologically similar to that induced by chlorphentermine.*** Light and electron microscopy confirmed the presence of "foam cells" in the alveolar spaces and their distribution throughout the lung tissue and elevated "foam cell" counts were found in both fenfluramine- and chlorphentermine-treated Wistar and BD9 rats.

(2) ***Fenfluramine induced the elevation of essentially the same phospholipids and, by implication, most likely via similar mechanisms compared to that induced by chlorphentermine in both Wistar and BD9 rats.*** This conclusion is drawn despite the observed presence of a strain

dependency seen for example in the Wistar rats being more susceptible to both fenfluramine and chlorphentermine treatment compared to the BD9 rats and differences in the levels of phospholipids in the lavaged and unlavaged lungs being observed.

In the unlavaged lungs fenfluramine, like chlorphentermine, induced the elevation of all the test phospholipids in the Wistar rats, except PG in the fenfluramine-treated and PG and PS in the chlorphentermine-treated rats. The PG and PS however only constituted a small percentage of the total lung phospholipids and the combined evidence was insufficient to conclude that there was a difference in the effects of the two drugs. Similarly in the unlavaged lungs of the BD9 rats, fenfluramine, like chlorphentermine, also induced the elevation of all the test phospholipids, except PS, PG and PA in both fenfluramine- and chlorphentermine-treated rats. Again it must be stated that the PS, PG and PA only constituted a small percentage of the total lung phospholipids and the combined evidence was insufficient to conclude that there was a difference in the effects of the two drugs.

In all cases lavaging of the lung significantly lowered the level of phospholipids present in the lung, confirming that most of the accumulation occurs in the alveolar spaces and macrophages.

(3) ***Only subtle differences exist in the mechanism of phospholipidosis induced by fenfluramine and chlorphentermine.*** This conclusion is supported by:

(a) the finding that essentially similar phospholipids were induced by both fenfluramine and chlorphentermine in the rats, and (b) the inconclusiveness of the data to clearly indicate whether the accumulation of phospholipids may occur exclusively via either direct inhibition or phospholipid-substrate complex- mediated inhibition of the phospholipases.

In the case of fenfluramine the inhibition of PL-A activity could be ascribed exclusively to neither direct inhibition of the enzyme nor reduced susceptibility of the phospholipid substrate-drug complex, while the PL-C activity appeared to be inhibited to a greater extent via the phospholipid substrate-drug complex rather than by direct inhibition. On the otherhand chlorphentermine induced a small but significantly greater degree of inhibition of PL-A activity more via direct inhibition than via the phospholipid substrate-drug complex, while the PL-C activity appeared to be inhibited to a greater extent via phospholipid substrate-drug complexation rather than by direct inhibition.

Further, the phase transition temperature studies, which was undertaken to detect possible differences in the extent of drug-phospholipid binding of the two drugs, seemed to support the idea that, for both drugs in both Wistar and BD9 rats, the elevation of PC, PE, PI, PS, SPM and PA (except PA in the BD9 rats) in the unlavaged lungs can be the result of phospholipid substrate modification rather than direct inhibition.

Collectively the results tend to indicate that small differences in essentially similar mechanisms, could most likely explain the differences in the phospholipidosis induced by fenfluramine and chlorphentermine.

Finally, a major finding of this study is the confirmation that the alveolar spaces and macrophages are the major sites for drug-induced phospholipid accumulation. Since the alveolar spaces are crucial sites for gas exchange, and the macrophages part of an essential protective system in the healthy lung, changes in the lung phospholipid composition, may thus be a useful early indicator of the respiratory complications that have been found to occur in phospholipidosis. The effect of drug-induced phospholipidosis on respiratory mechanics and on the optimal functioning of the alveolar macrophages needs to be further investigated. A preliminary study on the effect of phospholipidosis on respiratory mechanics has just been completed[91].

SUMMARY

SUMMARY

The aim of this study was to investigate the mechanism of fenfluramine-induced pulmonary phospholipidosis, by comparing the profile and levels of induced phospholipids in the rat and the mode of phospholipase inactivation, both relative to that produced by chlorphentermine.

Wistar and BD9 rats were injected with fenfluramine (FF) and chlorphentermine (CP) intra-peritoneally daily over a six week period to induce phospholipidosis. The lungs isolated from such treated and untreated animals, were grouped into unlavaged lungs and lungs to be lavaged and from the latter group the alveolar macrophages were isolated. Small sections of the unlavaged lungs were microscopically examined to verify the induction of phospholipidosis. Further the levels of phosphatidyl choline (PC), spingomyelin (SPM), phosphatidyl ethanolamine (PE), phosphatidyl glycerol (PG), phosphatidyl inositol (PI), phosphatidyl serine (PS) and phosphatidic acid (PA) were determined in both groups of lungs using a TLC method. To assess whether the drug-mediated inactivation of the phospholipases (PL) occurred via direct inhibition of the enzymes or via the drug-phospholipid complex, the hydrolysis of the above phospholipids by PL-A or PL-C were monitored using colorimetric methods. The feasibility of the phospholipid-drug complex-mediated mechanism was further explored, by assessing the effect the two drugs had on the phase transition temperature of the phospholipids.

Electron microscopy revealed the presence of hypertrophied and elevated counts of alveolar macrophages in the treated-Wistar and -BD9 rats. In the FF- and CP-treated Wistar and BD9 rats there were, compared to the saline-treated rats, a 200 % and 235 % increase in macrophage counts, respectively, for the FF-treated rats and a 700 % and 965 % increase in macrophage counts, respectively, for the CP-treated rats. The levels of all the phospholipids in the unlavaged lungs of both rat strains were elevated, except that for PG, PS and PA. In both rat strains following the treatment with both drugs the PG levels were not elevated and the PS levels

were not elevated following CP treatment. Following the treatment for both drugs, the PA levels were also not elevated in the BD9 rats. Relative to the levels found in the unlavaged lungs of the control rats, the increases ranged from a minimum of 9 to a maximum of 216 %. In general, Wistar rats appeared to be more susceptible to both FF and CP treatment. In both rat strains, lavaging of the lungs considerably reduced the levels of phospholipids remaining in the lung and the differences between the treated and untreated animals became less striking. The addition of FF or CP, whether directly to the enzyme, or in the form of the drug-phospholipid complex, resulted in significant decreases in the PL-A-mediated or PL-C-mediated hydrolysis of virtualy all the test phospholipids. The average decrease ranged from 0.811 to 4.04 μg FFA[bbb] μl^{-1} sample min^{-1}, for the PL-A activity and 0.023 to 0.827 μg IP[ccc] 100 μl^{-1} sample min^{-1}, for the PL-C activity. In the case of FF, the inhibition of PL-A activity could not be ascribed exclusively to either direct inhibition of the enzyme or reduced susceptibility of the phospholipid substrate-drug complex. The PL-C activity appeared to be inhibited to a greater extent via the phospholipid substrate-drug complex rather than by direct inhibition. On the other hand, CP induced a small, but significantly greater degree of inhibition of PL-A activity, more via direct inhibition, rather than by the phospholipid substrate-drug complex. The PL-C activity appeared to be inhibited to a greater extent via phospholipid substrate-drug complexation than by direct inhibition. From the above data, considered collectively, it was not possible to declare either of the two possible mechanisms as the more likely one for FF or CP-induced inhibition of the phospholipases. The feasibility of the indirect mode was further explored, by determining the phase transition temperatures for the phospholipid-drug complexes of each drug. The addition of each drug caused a

---

[bbb] FFA = free fatty acid

[ccc] IP = inorganic phosphate

depression of the phase transition temperature of all the phospholipids with a ΔT^{ddd} ranging from 0.52 to 15.73 °C. This appears to support the notion that both drugs bind to the phospholipids and the differences in the extent of the phase transition temperature depression of the individual phospholipids may indicate differences in the binding capacities of these drugs.

The following major conclusions may be drawn from the results of this investigation. Fenfluramine induces a phospholipidosis syndrome in the lungs of Wistar and BD9 rats that are histologically similar to that induced by CP. It induces the elevation of essentially the same phospholipids as CP, primarily in the alveolar spaces and macrophages, and by implication, most likely via similar mechanisms. For both FF and CP, both direct inhibition and phospholipid-drug complex-mediated inhibition of phospholipases were found to be a viable mechanism for this syndrome. The mechanism for FF-induced pulmonary phospholipidosis thus appears to be similar to that of CP; small quantitative differences in essentially similar mechanisms, may explain the differences in the levels of induced phospholipidosis found in this study.

---

ddd ΔT = The difference in the phase transition temperature between the phospholipid control and the phospholipid-drug complex.

LIST OF REFERENCES

LIST OF REFERENCES

1. LüLLMAN-RAUCH, G.H., REIL, G.H., ROSSEN, E. and SEILER, K.U. The ultrastructure of rat lung changes induced by an anoretic drug (chlorphentermine). Virchows Arch. B. Zellpath. 11, 167-181 (1972).

2. LüLLMAN, H., LüLLMAN-RAUCH. R., WASSERMAN, O. Drug-induced phospholipidosis. Germ. Med. 3, 128 (1973).

3. REASOR, M.J. Drug-induced lipidosis and the alveolar macrophage. Toxicology. 20, 1-33 (1981).

4. REASOR, M.J., KOSHUT, R.A. and CASTRANOVA, V. Biochemical characteristics of rat alveolar macrophages with chlorphentermine-induced phospholipidosis. Exp. Mol. Pathol. 31, 297 (1979).

5. LüLLMAN, H., LüLLMAN-RAUCH, R. and WASSERMAN, O. Lipidosis induced by amphiphilic cationic drugs. Biochem. Pharmacol. 27, 1103-1108 (1978).

6. BLOHM, T.R. Drug-induced lysosomal lipidosis: Biochemical

interpretations. Pharmacol. Rev. 30 (4), 593-603 (1979).

7. MARTIN II, W.J., KACHEL, D.L., VILEN, T. and NATARAJAN, V. Mechanism of phospholipidosis in Amiodarone pulmonary toxicity. J. Pharmacol. and Exp. Ther. 251 (1), 272-278 (1989).

8. VIJEYARATNAM, G.S. and CORRIN, B. Pulmonary histiocytosis simulating desquamative interstitial pneumonia in rats receiving oral iprindole. J. Pathol. 108, 105-113 (1972).

9. BASSETT, D.J.P., FISHER, A.B. and RABINOWITZ, J.L. Effect of hypoxia on incorporation of glucose carbons into lipids by isolated rat lung. Am. J. Physiol. 227, 1103-1108 (1974).

10. MORGAN, T.E., FINLEY, T.N. and FIALKOW, H. Comparison of the composition and surface activity of alveolar and whole lung lipids in the dog. Biochem. Biophys. Acta. 106, 403-413 (1965).

11. NAKUMURA, M. and ONODERA, T. Characteristics of phospholipids in human lung carcinoma. Lipids. 15, 616-623 (1980).

12. HARWOOD, J.L. Lung surfactant. Prog. Lipid Res. 26, 211-256 (1987).

13. HAAGSMAN, H.P., VAN GOLDE, L.M.G. Synthesis and assembly of lung surfactant. Annu. Rev. Physiol. 53, 441-64 (1991).

14. POSSMAYER, F. A proposed nomenclature for pulmonary surfactant-associated proteins. Am. Rev. Respir. Dis. 138, 990-998 (1988).

15. WEAVER, T.E. and WHITSETT, J.A. Function and regulation of expression of pulmonary surfactant-associated proteins. J. Biochem. 273, 249-264 (1991).

16. ZELIGS, B.J, NERURKAR, L.S. and BELLANTI, J.A. Maturation of the rabbit alveolar macrophage during animal development 1. Perinatal influx into alveoli and ultrastructural differentiation. J. Pediatr. Res. 11, 197-208 (1977).

17. SCHWARTZ, L.W. and CHRISTMAN, C.A. Alveolar macrophage migration - Infleunce of lung lining material and acute lung insult. Am. Rev. Resp. Dis. 120 (2), 429-439 (1979).

18. BHAGAVAN, N.V. Biochemistry - A comprehensive review. J.B. Lippincott Co. (1973).

19. MARTIN, D.W.Jr, MAYES, P.A., RODWELL, V.W. and GRANNER, D.K. Harper's review of biochemistry (20th Edition) Lange medical publications, California, USA (1983).

20. HOSTETLER, K.Y. and MATSUZAWA, Y. Studies on the mechanism of drug induced lipidosis. Cationic amphiphilic drug inhibition of lysosomal phospholipases-A and -C. Biochem. Pharmacol. 30 (10), 1121-1126 (1981).

21. HOSTETLER, K.Y. Molecular studies of the induction of cellular phospholipidosis by cationic amphiphilic drugs. Fed. Proc. 43, 2582-2585 (1984).

22. WARREN, K.R., SCHAFFER, J.A., SULLIVAN, J.C., PETRELLI, M. and RADIN, N.S. The effects of N-hexyl-O-glucosyl sphingosine on normal cultured human fibroblast: a chemical model for Gaucher's disease. J. Lipid. Res. 17, 132-138 (1970).

23. SAKURAGAWA, N., SAKURAGAWA, M., KUWABARA, T., PENTCHEV, P.G., BARRANGER, J.A. and BRADY, R.O. Niemann-Pick disease experimental model: sphingomyelase reduction by AY-9944. Science. 196, 317-319 (1977).

24. KASAMA, K., YOSHIDA, K., TAKEDA, S., AKEDA, S. and KAWAI, K. Bis-(monoacylglyceryl) phosphate and acyl phosphatidylglycerol isolated from human livers of lipidosis by 4,4'-diethylaminoethoxyhexestrol. Lipids 11, 712-721 (1976).

25. YAMAMOTO, A., ADACHI, S., ISHIKAWA, K., YOKOMURA, T., KITANI, T., NASU, T., IMOTO, T. and NISHIKAWA, M. Studies on drug-induced lipidosis. III. Lipid composition of the liver and some other tissues in clinical cases of " Nieman-Pick-like syndrome " induced by 4,4' diethylaminoethoxyhexestrol. J. Biochem. 70, 775-784 (1971).

26. DE LA IGLESIA, F., FEUER, G., McGUIRE, E.J. and TAKADA, A. Morphological and biochemical changes in the liver of various species in

experimental phospholipidosis after diethylaminoethoxyhexestrol treatment. Toxicol. Appl. Pharmacol. 34, 28-44 (1975).

27. KACEW, S. Gentamicin or chlorphentermine induction of phospholipidosis in the developing organism: role of tissue and species in manifestation of toxicity. J. Pharmacol. and Exp. Therapeutics. 232 (1), 239-243 (1985).

28. LüLLMAN, H and WEHLING, M. The binding of drugs to different polar lipids in vitro. Biochem. Pharmacol. 28, 3409-3415 (1979).

29. MILES, P.R., BOWMAN, L., TUCKER, J., REASOR, M.J., and WRIGHT, J.R. Alterations in rat alveolar surfactant phospholipids and proteins induced by administration of chlorphentermine. Biochim. Biophys. Acta. 877, 167-178 (1986).

30. JOSHI, U.M., KODAVANTI, P.R.S., COUDERT, B., DWYER, T.M. and MEHENDALE, H.M. Types of interaction of amphiphilic drugs with phospholipid vesicles. J. Pharmacol. and Exp. Therapeutics. 246 (1), 150-157 (1988).

31. MATSUZWA, Y. and HOSTETLER, K.Y. Inhibition of lysosomal phospholipase-A and phospholipase-C by chloroquine and 4,4' bis(diethylaminoethoxy)α,β-diethyldiphenylethane. J. Biol. Chem. 255 (11), 5190-5194 (1980).

32. YAMAMOTO, A., ADACHI, S., MATSUZWA, Y., KITANI, T., HIRAOKA, A. and SEKI, K. Studies on drug-induced lipidosis. Effects of lipid composition of bis-λ-diethylaminoethylether of hexestrol, chloroquine, homochlorocyclizine, prenylamine and diazacholestrol on the lipid composition of rat and kidneys. Lipids 11, 616-622 (1976).

33. KEHRER, J.P. and KACEW, S. Systematically applied chemicals that damage lung tissue. Toxicology 35, 251-293 (1985).

34. KARABELNIK, D. and ZBINDEN, G. Drug-induced foam cell reactions in rats. II. Chemical analysis of lipids stored in lungs and foam cells after treatment with chlorphentermine, 5-[p-(fluoren-9-ylidenemethyl)phenyl]-2-piperidine-ethanol(RMI IO, 393) and 1-chlor-amitriptyline. Hoppe-Seyler's Z. Physiol. Chem. 356, 1151-1160 (1975).

35. WILSON, B.D., CLARKSON, C.E. and LIPPMANN, M.L. Amiodarone-induced pulmonary inflammation: correlation with drug dose and lung levels of drug, metabolite and phospholipid. Am. Rev. Respir. Dis. 143, 1110-1114 (1991).

36. STERN, N., TIETZ, A., GATON, E. and WOLMAN, M. Effects of chlorocyclizine on pulmonary lipid metabolism in rats. Biochimica et Biophysica Acta. 754, 166-173 (1983).

37. Martindale, The Extra Pharmacopoeia. (28th Edition). The Pharmaceutical Press. (1982).

38. LüLLMAN, H., ROSSEN, E. and SEILER, K.U. Chlorphentermine in chronically treated rats. J. Pharm. Pharmac. 25, 239 (1973).

39. HEYMAN, C.A. and REASOR, M.J. Role of the alveolar macrophage in the induction of pulmonary phospholipidosis by chlorphentermine. II. Drug uptake into cells in vitro. J. Pharmacol. and Exp. Ther. 236 (1), 60-64 (1886).

40. CALDWELL, J. The metabolism of amphetamines in mammals. Drug Metab. Rev. 5 (2), 219 (1976).

41. KARABELNIK, D., ZBINDEN, G. and BAUMGARTNER, E. Drug-induced foam cell reactions in rats I. Histopathologic and cytochemical observations after treatment with chlorphentermine, RMI 10.393 and Ro 4-4318. Toxicol. Applied Pharmacol. 27, 395-407 (1974).

42. KACEW, S., NARBAITZ, R. and DUBAS, T.C. Biochemical and morphologic investigation of the influence of chlorphentermine and subsequent withdrawal on newborn rat lung. Toxicol. Appl. Pharmacol. 47, 185 (1979).

43. SEILER, K.U. and WASSERMAN, O. Drug-induced phospholipidosis II. Alterations in the phospholipid pattern of organs from mice, rats and guinea-pigs after chronic treatment with chlorphentermine. Naunyn-Schmiedeberg's Archs Pharmac. 288, 261-268 (1975).

44. PINDER, R.M., BROGDEN, R.N., SAWYER, P.R., SPEIGHT, T.M. and AVERY, G.S. Fenfluramine: A review of its pharmacological properties and therapeutic efficacy in obesity. Drugs. 10, 241 (1975).

45. LüLLMAN-RAUCH, R. and REIL, G. Fenfluramine-induced ultrastructural alterations in tissue of rats and guinea pigs. Naunyn-Schmiedeberg's Arch. Pharmacol. 285, 175-184 (1974).

46. BECKETT, A.H. and SALMON, J.A. pharmacokinetics of absorption, distribution and elimination. J. Pharm. Pharmac. 24, 108 (1972).

47. TISCHNER, K. Effects of chloroquine on neurons of long-term cultures of peripheral and central nervous system. A light and electron microscopic study. Acta Neuropath. (Berl) 28 (3), 233-242 (1974).

48. MARTIN II, W.J., KACHEL, D.L., VILEN, T. and NATARAJAN, V. Mechanism of phospholipidosis in amiodarone pulmonary toxicity. J. Pharmacol. and Exp. Ther. 251 (1), 272-278 (1979).

49. LüLLMAN-RAUCH, R. Lipidosis-like ultrastructural alterations in rat lymph nodes after treatment with tricyclic antidrepressants or neuroleptics. Naunyn-Schniedeberg's Arch. Pharmacol. 286, 165-179 (1974).

50. DIETERT, S.E. and SCALLEN, T.J. An ultrastructural and biochemical study of the effects of three inhibitors of cholesterol biosynthesis upon murine adrenal gland and testis. Histochemical evidence for a lysosome response. J. Cell Biol. 40, 44-60 (1969).

51. RADIN, N. Extraction of tissue lipids with a solvent of low toxicity. Methods in Enzymology 72, 5-7 (1981).

52. FOLCH, J. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226, 497-509 (1957).

53. VALODIA, P. Selected pulmonary adverse effects induced by fenfluramine in the rat -A comparison with chlorphentermine. M.Pharm Thesis, UWC (1989).

54. CLARKE, E.G.C. Isolation and identification of drugs. The Pharmaceutical Press. (1969).

55. SITTIG MARSHALL. Pharmaceutical manufacturing Encyclopedia. Noyes Data Corporation. pg.140 (1979).

56. HUMASON. G.L. Animal Tissue Technique. W.H. Freeman and Co. (1970).

57. BAKER, F.J. and SILVERTON, R.E. Introduction to Medical Laboratory Technology. (5th Edition) Butterworth Scientific Publ. (1980).

58. KIRK, L.J.C., PEEL, R.N., JAMES, K.R., LEWIS, K.S. and WAFT, A.D. Basic Medical Lab. Technology. (Second Edition) Pitman Books Ltd. (1982).

59. CARLETON, E.A. Histological Technique. (Fourth edition) Oxford University Press (1973).

60. HAYAT, M.A. Principles and techniques of scanning electron miscropy. Van Nostrand Reinold Company.

61. Instruction manual for the Coulter® counter, Model M530. Coulter® Electronic Limited.

62. REASOR, M.J. Cumene hydroperoxide-mediated lipid peroxidation in rat alveolar macrophages following induction of phospholipidosis with chlorphentermine. Toxicology. 18, 159-168 (1980).

63. McNULTY, M.J. and REASOR, M.J. Iprindole-induced phospholipidosis in rat alveolar macrophages - Alterations in oxygen consumption and release of oxidants. Exp. Lung Res. 2, 57-69 (1981).

64. ROUSER, G. and FLEISCHER, S. Isolation, characterisation and determination of polar lipids of the mitochondria. Methods in enzymology. 10, 385-406 (1967).

65. ENTEZAMI, A.A., VENABLES, B.J. and DAUGHERTY, E. Analysis of lipids by one-dimensional thin layer chromatography. J. Chrom. 387, 323-331 (1987).

66. MACALA, L.J., ROBERT,K. Yu. and ANDO, S. Analysis of brain lipids by high performance thin layer chromatography and densitometry. J. Lipid Research. 24, 1243-1250 (1983).

67. HEDEGAARD, E. and JENSEN, B. Nano-scale densitometric quantitation of phospholipids. J. Chrom. 225, 450-454 (1981).

68. KRUG, E.L. and KENT, C. Assay for phopholipase C. Methods in Enzymology. 72 347-351 (1981).

69. HENDRIKSON, H.S. and DENNIS, E.A. Kinetic analysis of the dual phospholipid model for phospholipase-A_2. J. Biol. Chem. 259 (9), 5734-5739 (1984).

70. LAUWERYS, R.R. Colorometric determination of free fatty acids. Anal. Biochem. 32, 331-333 (1969).

71. KRUG, E.L., TRUESDALE, N.J. and KENT, C. A simplified assay for phospholipase C. Anal. Biochem. 97, 43-47 (1979).

72. DALY, J.A. and ERTINGSHASEN, G. Direct mrthod for determining inorganic phosphate in serum with the "Centrifichem". Clin. Chemistry 18 (3), 263-265 (1972).

73. GAMST, O. and TRY, K. Determination of serum-phosphate without deproteinization by ultraviolet spectrophotometry of the phosphomolbdic acid complex. Scand. J. Clin. Lab. Invest. 40, 483-486 (1980).

74. MOHR, K. and STRUVE, M. Differential influence of anionic and cationic charge on the ability of amphiphilic drugs to interact with DPPC-liposomes. Biochem. Pharmacol. 41 (6), 961-965 (1991).

74. TRULSON, M.E. and JACOBS, B.L. Behavioural evidence for the rapid release of CNS serotonin and fenfluramine. Eur. J. Pharmacol. 36, 149 (1976).

75. RODNEY, F.M., ILETT, K.F. and MADSEN, B.W. A compartmental model for the uptake of chlorphentermine in isolated perfused rat lung. Eur. J. Drug Metab. and Pharmacokinetics. 6 (2), 127-133 (1981).

76. TAKEMURA, T., ROM, W.N., FERRANS, V.J. and CRYSTAL, R.G. Morphologic characterization of alveolar macrophages from subjects with occupational exposure to inorganic particles. Am. Rev. Respir. Dis. 140 1674-1685 (1989).

77. ROSEN, E., SEILER , K.U. and WASSERMAN, O. On a striking pharmacokinetic difference between chlorophentermine and phentermine. Naunyn-Schmiedeberg's Arch. Exp. Path. Pharmak. 274, R94 (1972).

78. KACEW, S. and NARBAITZ, R. A comparative ultrastructural and biochemical study between the effects of chlorphentermine and phentermine on rat lung. Exp. Mol. Path. 27, 106 (1977).

79. VAN FURTH, R. Mononuclear Phagocytes: Functional Aspects Part 1. Martinus Nijhoff Publishers (1980).

80. REASOR, M.J., KOSHUT, R.A., McNULTY, M.J. and TRUSH, M.A. Chemiluminescence from rat alveolar macrophages following induction of phospholipidosis with chlorphentermine. Toxicol. Appl. Pharmacol. 52 (3), 497-506 (1980).

81. ROMEO, D., ZABUCCHI, G., SORANZO, M.R. and ROSSI, F. Macrophage metabolism - activation of NADPh oxidation by phagocytosis. Biochem. Biophys. Res. Commun., 45, 1056 (1971).

82. KACEW, S and REASOR, M.J. Chlorphentermine-induced alterations in pulmonary phospholipid content in rats. Biochem. Pharmacol. 32 (18), 2683-2688 (1983).

83. SEYDEL, J.K. and WASSERMAN. O. NMR-studies on the molecular basis of drug induced phospholipidosis - II. Interaction between several amphiphilic drugs and phospholipids. Biochem. Pharmacol. 25, 2357-2364 (1976).

84. CATER, B.R., CHAPMAN, D., HAWES, M. and SAVILLE, J. Lipid phase interactions and drug interactions. Biochemica and Biophysica Acta, 363, 54-69 (1974).

85. DATTA, D.B. A comprehensive introduction to membrane biochemistry. Floral Publishing, New York (1989).

86. HANPFT, R. and MOHR, K. Influence of cationic amphiphilic drugs on the phase-transition temperature of phospholipids with different polar

headgroups. Biochimica et Biophysica Acta 814, 156-162 (1985).

87. KURSCH, B., LüLLMANN, H. and MOHR, K. Influence of various cationic amphiphilic drugs on the phase transition temperature of phosphatidylcholine liposomes. Biochem. Pharmacol. 32 (17) 2589-2594 (1983).

88. HALLMAN, M., SPRAGG, R., HARRELL, J.H., MOSER, K.M. and GLUCK, L. Evidence of lung surfactant abnormality in respiatory failure. Study of bronchoalveolar lavage phospholipids, surface activity, phospholipase activity, and plasma myoinositol. Jnl Clin. Invest. 70, 673-683 (1982).

89. KING, R.J., COALSON, J.J., SEIDENFELD, J.J., ANZUETO, A.R., SMITH, D.B. and PETERS, I.J. O^2- and pneunonia induced lung injury II. Properties of pulmonary surfactant. J. Appl. Physiol. 67 (1), 357-365 (1989).

90. KING, J.R., COALSEN, J.J., SEIDENFELD, J.J., ANZUETO, A.R., SMITH, B.D. and PETERS, J.I. Oxygen and pneumonia-induced lung injury II. Properties of pulmonary surfactant. J. Appl. Physiol. 67(1), 357-365 (1989).

91. HOFFMESTER, N. The effect of drug-induced pulmonary phospholipidosis on dynamic mechanical lung function and perfusate gas composition in the isolated perfused rat and guinea-pig lungs. M.Pharm.

CURRICULUM VITAE

CURRICULUM VITAE

NAME: Mogamat Shafick Hassan

SEX: Male

DATE OF BIRTH: 23 February 1960

ADDRESS: 20 Olivine Road
Rondebosch East
7700

TELEPHONE NUMBER: 6962621/6960511 (HOME)
9596272 (WORK)

QUALIFICATIONS:

Academic: Matriculated from Alexander Sinton High School-(1977)
Bachelor of Pharmacy (B.Pharm) U.W.C. - 1983

Professional: Pharmacist - Registered with the S.A.Pharmacy Council.

Professional Affiliations: None

Other: Member of Antartic Philatelic Society.

PAPERS DELIVERED:

(a) Electron Microscopy of foam cell formation after chronic treatment with chlorphentermine and fenfluramine.

S.A. Academy of Pharmaceutical Sciences, Cape Town. (May 1986)

(b) Pulmonary phospholipid levels following chronic chlorphentermine and fenfluramine administration in rats.

S.A. Pharmacological Society, Durban. (October 1991)

PUBLICATIONS: B. Burger and M.S. Hassan, Co-author - Fundamentals of Laboratory Pharmacology (1989).