A RAPID METHOD FOR THE PREPARATION OF LUNG CELL FRACTIONS
ENRICHED IN ALVEOLAR TYPE II PNEUMOCYTES

by

Walter Mark Lafranconi

A Thesis Submitted to the Faculty of the
COMMITTEE ON TOXICOLOGY (GRADUATE)
In Partial Fulfillment of the Requirements
For the Degree of
MASTER OF SCIENCE
In the Graduate College
THE UNIVERSITY OF ARIZONA

1981
STATEMENT BY AUTHOR

This thesis has been submitted in partial fulfillment of requirements for an advanced degree at The University of Arizona and is deposited in the University Library to be made available to borrowers under rules of the Library.

Brief quotations from this thesis are allowable without special permission, provided that accurate acknowledgment of source is made. Requests for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the head of the major department or the Dean of the Graduate College when in his judgment the proposed use of the material is in the interests of scholarship. In all other instances, however, permission must be obtained from the author.

SIGNED: W. [Signature]

APPROVAL BY THESIS DIRECTOR

This thesis has been approved on the date shown below:

KLAUS BRENDEL
Professor of Pharmacology

[Signature]
April 29, 1981
Date
This work is dedicated to my parents

Walter and Allitta Lafranconi

and my sister

Kay Lafranconi
ACKNOWLEDGMENTS

I would like to thank Dr. Klaus Brendel for his insight, enthusiastic direction and unwavering encouragement during this project. I would also like to thank Dr. Glenn Sipes for his careful guidance and helpful suggestions. Without these two gentlemen this work never would have been completed.

I would further like to thank Dr. Dick Spall and Dr. Ray Duhamel for the many occasions when they took time from their own research to help me.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF ILLUSTRATIONS</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>viii</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>ix</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>Statement of Purpose</td>
<td>25</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>30</td>
</tr>
<tr>
<td>Animals</td>
<td>30</td>
</tr>
<tr>
<td>Buffers</td>
<td>30</td>
</tr>
<tr>
<td>CaCl₂ and MgCl₂ Solutions</td>
<td>30</td>
</tr>
<tr>
<td>EGTA Solutions</td>
<td>31</td>
</tr>
<tr>
<td>EDTA Solution</td>
<td>31</td>
</tr>
<tr>
<td>Magnetite Preparation (Fe₃O₄)</td>
<td>31</td>
</tr>
<tr>
<td>Trypsin Solutions</td>
<td>32</td>
</tr>
<tr>
<td>Trypsin Inhibitor DNAs Solutions</td>
<td>32</td>
</tr>
<tr>
<td>Thermolysin Solutions</td>
<td>32</td>
</tr>
<tr>
<td>Collagenase Solutions</td>
<td>33</td>
</tr>
<tr>
<td>Elastase Solutions</td>
<td>33</td>
</tr>
<tr>
<td>Pronase Solution</td>
<td>33</td>
</tr>
<tr>
<td>Karnovsky's Fixative</td>
<td>33</td>
</tr>
<tr>
<td>Trypan Blue Stain</td>
<td>34</td>
</tr>
<tr>
<td>Reagents for Papanicolaou Stain</td>
<td>34</td>
</tr>
<tr>
<td>Harris's Hematoxylin</td>
<td>34</td>
</tr>
<tr>
<td>Lithium Carbonate</td>
<td>34</td>
</tr>
<tr>
<td>Orange G and EA-50</td>
<td>35</td>
</tr>
<tr>
<td>Ethanol Washes</td>
<td>35</td>
</tr>
<tr>
<td>Xylene Ethanol Wash</td>
<td>35</td>
</tr>
<tr>
<td>Acridine Orange Fluorescent Stain</td>
<td>35</td>
</tr>
<tr>
<td>Osmium Tetroxide Solution for Lipid Reaction</td>
<td>35</td>
</tr>
<tr>
<td>O₂ Measuring Equipment</td>
<td>35</td>
</tr>
<tr>
<td>Preparation of Lung</td>
<td>36</td>
</tr>
<tr>
<td>Enzymatic Dispersion of Lung Matrix</td>
<td>38</td>
</tr>
<tr>
<td>Removal of Cells, Method 1</td>
<td>39</td>
</tr>
<tr>
<td>Removal of Cells, Method 2</td>
<td>39</td>
</tr>
<tr>
<td>Magnetic Removal of Macrophages</td>
<td>40</td>
</tr>
<tr>
<td>Cell Counting</td>
<td>40</td>
</tr>
</tbody>
</table>
### TABLE OF CONTENTS—Continued

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Viability -- Trypan Blue Exclusion</td>
<td>43</td>
</tr>
<tr>
<td>O2 Uptake</td>
<td>43</td>
</tr>
<tr>
<td>14C Acetate and 14C Choline Uptake</td>
<td>44</td>
</tr>
<tr>
<td>Cell Identification</td>
<td>45</td>
</tr>
<tr>
<td><strong>RESULTS</strong></td>
<td>47</td>
</tr>
<tr>
<td>Evolution of Procedure</td>
<td>47</td>
</tr>
<tr>
<td>Enzymatic Dispersion of Lung Matrix</td>
<td>47</td>
</tr>
<tr>
<td>Characterization of Cells</td>
<td>67</td>
</tr>
<tr>
<td>Cell Yield and Purity</td>
<td>67</td>
</tr>
<tr>
<td>Cell Viability</td>
<td>70</td>
</tr>
<tr>
<td>Cell Identity</td>
<td>73</td>
</tr>
<tr>
<td><strong>DISCUSSION</strong></td>
<td>81</td>
</tr>
<tr>
<td>Future Work</td>
<td>89</td>
</tr>
<tr>
<td><strong>APPENDIX A: METHOD FOR MODIFIED PAPANICOLAOU STAIN</strong></td>
<td>91</td>
</tr>
<tr>
<td><strong>APPENDIX B: FLOW DIAGRAM OF PROCEDURE</strong></td>
<td>93</td>
</tr>
<tr>
<td><strong>REFERENCES</strong></td>
<td>95</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

Figure | Page
---|---
1. Recirculatory Perfusion Apparatus | 37
2. Separation Chamber | 41
3. Cell Separator | 42
4. Effect of Time on Yield of Viable Pneumonocytes | 54
5. Transmission Electron Micrograph of Fe$_3$O$_4$ Containing Macrophage | 60
6. Effect of Time on Uptake of Magnetite by Pulmonary Macrophages | 62
7. Magnetic Probe before Addition of Macrophages | 63
8. Magnetic Probe Immediately after Addition of Macrophages | 64
9. Magnetic Probe 1 Minute after Addition of Macrophages | 65
10. Removal of Macrophages Containing Fe$_3$O$_4$ as Measured by Decreased Absorbance at 600 nm | 66
11. Comparison of Yield and Purity between Methods 1 and 2 | 69
12. Oxygen Consumption of Isolated Type II Cells | 72
13. Incorporation of Acetate and Choline into Disaturated Lecithins | 74
14. Transmission Electron Micrograph of Isolated Type II Cells in a Field of 3 Cells | 75
15. Modified Papanicolaou Stained Cells | 76
16. Fluorescence Microscopy of Type II Cell Preparation | 77
17. Transmission Electron Microscopy of an Isolated Type II Cell | 78
LIST OF TABLES

Table                                                Page

I. Comparison of Methods for Isolating Type II Cells   26

II. Effectiveness of Various Proteases on Dispersion of Lung Parenchyma from Matrix 48

III. Effect of 10 Minute Incubation on Cell Viability 52

IV. Effects of Various Routes of Administration of Enzymes on Yield of Viable Type II Cells 55

V. Major Cell Types Released from Lung Matrix 57

VI. Effect on Cell Population Profile of Sizing through a 15 m Nylon Mesh Filter 59

VII. Summary of Characteristics of Cells Isolated by Recirculatory Perfusion, Sizing and Magnetic Macrophage Removal 68

VIII. Relative Nature of Contaminants in Purified Type II Preparations 71

IX. Elutriator Cell Fraction Analysis of Isolated Type II Cells 80
ABSTRACT

A method was developed for rapid isolation of type II granular pneumocyte fractions by the use of magnetic iron oxide to afford removal of macrophages. In this procedure, isolated lungs from white New Zealand rabbits perfused with tissue culture medium, were lavaged with colloidal magnetite. Recirculatory perfusion of collagenase and elastase enzymatically dispersed alveolar areas while removing endothelial cells from the lung. Macrophages, having phagocytosed the magnetite particles, were pulled out of the mixture in a strong magnetic field. Sizing of the macrophage-depleted pneumocyte suspension yielded an enriched type II cell preparation. Viability of purified cell suspension was 90-95% as determined by trypan blue exclusion. Cellular oxygen uptake measured with Clarke type $O_2$ electrode was $88 \pm 6 \text{ nM } O_2/10^6 \text{ cells/hr}$. Isolated cells linearly incorporated $^{14}C$ acetate and $^{14}C$ choline at $0.44 \pm 0.1$ and $0.115 \pm 0.01 \text{ nM}/10^6 \text{ cells/hr}$, respectively. Granular pneumocytes (type II) were identified by modified Papanicolaou stain, fluorescence microscopy and electron microscopy. Yield of granular type II pneumocytes in this procedure as well as their relative purity were comparable to methods described in the literature. For pharmacological or toxicological investigations, this method provides rapid isolation of cells and eliminates the use of costly or specialized equipment.
INTRODUCTION

This work describes efforts to isolate alveolar type II cells from lungs. Methods available in the literature proved to be unsatisfactory. Therefore our goals were to develop a method which produced high yields and viability of cells as well as high purity and which would also be rapid, simpler and less costly than published procedures.

Initially, we attempted to duplicate the available methods for isolating septal cells. In our hands, these published procedures provided us with disappointing results. Cell yields, viability, and purity were all lower than reported. In addition, times required to complete these procedures proved prohibitive for any further experiments planned. We therefore decided to investigate and develop an original method to prepare type II cell fractions from mammalian lungs.

Before describing procedures, it is necessary to present some primary concepts regarding the lung in order to provide a basis for discussion of results and establish a reasoning for selection of methods. Concepts to be discussed include structure as it dictates function as well as some important physiological relationships relating to normal and stressed conditions within the lung.

There must also be justification for selecting certain cells for isolation. With over 40 cell types within the lung, why try to isolate the cells we did? To answer this, normal and abnormal function of important cell types will be described in order to demonstrate the
importance of various cells as they relate to lung function and more importantly as they respond to toxic insult.

The lung has a variety of functions important to regulation of the internal environment of the system. It is the primary site for gaseous exchange as well as functioning exocrine and endocrine roles. From a toxicological point of view, the lung is important to study since it is the primary route of entry to the system of many airborne contaminants. The pulmonary system is doubly jeopardized in that it is susceptible to damage from systemic routes in addition to the airborne insult.

The lung is comprised of over 40 cell types (1), each with its own functions and unique responses to injury. The study of the lung must, therefore, consider the alterations in the lung as an intact organ as well as alterations in important cell types within the lung that could lead to impaired pulmonary function of a more discrete nature.

The primary function of the lung is gaseous exchange between the internal and external milieu. In order to accomplish this, the lung must be structured to allow optimal gas exchange as well as structural strength to withstand the deformations that occur during ventilation and non-ordinary stress such as encountered in coughing or sneezing.

The actual mechanism for gas exchange has been under study for some time. In 1902, Bohr suggested secretion of gasses as the mechanism of exchange but it was later shown that passive diffusion was the main exchange process (2,3). Diffusion as it occurs in the lung is a rapid event dependent upon the factors outlined in Fick's law. In a normal
steady state mammalian system, $O_2$ diffusion requires 0.79 seconds to reach equilibrium while $CO_2$, being 20 times more diffusable, requires only 0.04 seconds \((4,5)\).

The function of gas exchange via diffusion has been formally described by Fick's First Law of gaseous diffusion \((5)\). A careful evaluation of this law with respect to the lung will emphasize the close adherence to the stipulations of the laws of diffusion that are evident in the construction of the lung. To utilize this model, we must first assume that:

1. The blood is perfectly mixed at all times,
2. Homogeneous with respect to the capacity of blood for the gas,
3. Totally isolated from blood undergoing similar exchanges in other alveoli,
4. The blood is flowing at a uniform rate rather than pulsing,
5. The alveolar gas is perfectly mixed.

As it turns out, the assumptions made above are not unreasonable and have been used by many workers in their investigations of lung gas diffusion. The added mathematical complexity required to more completely describe the system would prove to be too complicated for a general assessment of the lung gas diffusion and due to the natural biological variability of the systems would offer very little improved data accuracy. Fick's Law states:

$$V_x(t) = -D_x [P_{ax} - P_x(t)]$$
In equation 1 the left-hand side expresses the flow rate of the gas $x$ (ml/min) across the blood-gas barrier instantaneously at time $t$; $D_x$ represents the ability of the gas to diffuse across the blood-gas barrier (ml/min per mm Hg partial-pressure difference across the barrier). Thus, $D_x$ corresponds to the diffusing capacity; it is a constant at all points along the pulmonary capillary under the above assumptions. $P_{ax}$ is the uniform and time-invariant alveolar partial pressure (mm Hg), and $P_x(t)$ is the instantaneous capillary blood partial pressure (mm Hg). The negative sign accounts for the fact that, as equilibration proceeds, $P_x(t)$ approaches $P_{ax}$ so that the mass flow rate falls as time increases.

$D_x$ can be expressed in terms of its components. The constant of proportionality term is a composite term that is directly proportional to:

1. The surface area across which diffusion is occurring,
2. The solubility of the gas in the blood gas barrier.

This term is also inversely proportional to:

1. The thickness of the barrier,
2. The square root of the gas.

$$D_x = k \cdot \frac{A}{d} \cdot \frac{a_x}{\sqrt{MW_x}}$$

Here $k$ is the diffusion coefficient of the gas $x$ in the blood-gas barrier (cm²/min), $A$ is the cross-sectional area over which diffusion occurs (cm²), $d$ is the thickness of the blood-gas barrier (cm), $a_x$ is the solubility of the gas $x$ in the blood-gas barrier in ml gas/100 ml blood at standard temperature and pressure, and $MW_x$ is the molecular weight of
the gas. The variables $k$, $A$, and $d$ are properties of the blood-gas barrier; $a_x$ and $MW_x$ are properties of the particular gas under consideration.

Factors which alter any aspect of lung structure will affect relationships described by Fick's Law of gaseous diffusion. This point will be important because many pulmonary toxins do indeed affect membrane solubility, diffusion pathlengths, cross sectional areas in the alveolar capillary barrier and alveolar or capillary partial pressures.

Surface areas in the lung are large owing to the primary design of gas exchange requirements. The alveolar surface area is estimated to be $160 \text{ m}^2$ with a capillary surface area of $138 \text{ m}^2$ (6). It is further estimated the gas exchange pathway is less than $1.6 \mu\text{m}$ in thickness (6). The abundant surface area of both the capillary and alveolar aspect of the lung present a good locale for the gaseous exchange to take place in accordance with the $D_x$ term of Fick's Law.

The lung has a branching pattern of airways that minimizes airway resistance but more importantly ends in a respiratory zone where turbulent airflow is minimized in order to allow efficient gaseous diffusion. This area is termed the alveolar region. Here the alveolar ducts are surrounded by thin gas exchange membranes forming alveolar sacs. These sacs contain a lumen in which the gas from ventilation or respiration accumulates, a bilayer of surfactant, a layer of epithelial cells, a basement membrane followed by endothelial lining of capillaries (4). The diffusion across this barrier is related to time as well as surface area, diffusion path length and a concentration gradient as per
Fick's Law. The branching of the airways reduces airflow velocity which serves to provide adequate time for gas exchange to take place. Twenty-three major airway branchings take place which allow the reduction of turbulent airflow. The motion of the gas flow at this point is directed strictly by diffusion gradients and not by the mechanical movement of the air due to ventilation. The mean air velocity at the alveoli is 0.003 cm/sec.

The alveolar area is populated by 4 main cell types. Three of these cell types are closely connected to each other at tight junction zona occludens and rest on the basement membrane while the fourth type is capable of mobility and is not considered an integral structural component of the alveoli. This system of cells in the alveolus forms a relatively impermeable barrier to all but simple movement of small molecules (7).

In the alveolus, type I cells function primarily as gas exchange units. They cover 90% of the surface of the alveolus but only make up 10% of the alveolar cells by numbers and only 13% of the volume (8). They are the largest of the cells in the alveolus but are extremely thin. This thin flat structure provides a minimal path length thus augmenting diffusion as per the $D_x$ term in Fick's Law. The type I cells may possess squamous extensions intercollating with other cells forming zona occludens junctions. These extensions may extend as far as 50 μm from the nucleus (8). The thickness of the type I cells are remarkably consistent from species to species (8). The average value of thickness of human epithelial type I cells is 0.48 μm (9).
There are few inclusions in the cytoplasm of the cell. The nucleus is approximately 5 μm in diameter. The cell also contains small numbers of mitochondria, a primitive golgi apparatus and scant endoplasmic reticulum. The type I cells are not capable of mitosis following injury but are replaced by differentiated type II cells which evolve into type I cells and function as the primary gas exchange cell.

The second cell type in the alveolus, the type II cells, are cuboidal and actually more numerous than the type I cells, although they do not occupy the surface area of the alveolus as extensively as the type I cell. They represent 15-20% of the total cell numbers in the lung and 80% of the alveolar cell count (10). Type II cells are located in the corners of the alveolus and are the cells responsible for surfactant production and secretion (11). They have large irregular nuclei, many cytoplasmic organelles including numerous large mitochondria, a well-developed golgi apparatus and endoplasmic reticulum plus characteristically numerous large osmiophilic lamellar bodies (11). The surface of the cell contains micro-villi 0.1 μm in diameter and extrusions of the lamellar bodies from the cell into the extracellular spaces can be observed (10).

The most popularly accepted function of the type II cell is the secretion of surfactant. The characteristic lamellar bodies observed in the cells contain surfactant (11). The unfolding of these lamellar bodies extracellularly further suggest the function of the type II cell is surfactant production and secretion (12). It has been further shown that type II cells will rapidly take up the $^{14}$C-labeled precursors
of surfactant both in vivo and in vitro (13,14). Isolated type II cells also contain and synthesize phosphatidylglycerol and disaturated phosphatidylcholine -- two phospholipids found in surfactant that are rather uncommon in other cells (12).

A further role of type II cells is their function in response to pulmonary injury. Type I cells, due to their thin and vulnerable nature are most susceptible to damage and, as previously reported, are unable to undergo mitosis for homeostatic repair (15). Type I cells may die and slough off into alveolar spaces in response to acute lung injury. The alveolar type II cells, which are more resistant to injury, proliferate to re-establish a continuous epithelium (15,16). Cytokinetic studies in the rat have demonstrated that type II cells can develop into type I cells after pulmonary injury from NO₂ or oxygen (17,18). It has been shown that type II cells can develop into type I cells during normal differentiation in the fetal rat lung (19). Thus, some have suggested that the type II cells are the stem cells from which all the other epithelial cells of the lung develop. So far, only type II cell to type I cell differentiation has been demonstrated. Other cell types in the lung appear to have developed from cells precursory to type II (20).

It has further been shown that type II cells can respond to hormones as well as drugs and other xenobiotics. Dexamethasone (21), Betamethasone (22), estrogens (23), and thyroid hormone (24) have been shown to increase surfactant synthesis by type II cells. In addition, heroin, caffeine and theophylline have been shown to increase surfactant
synthesis while phenobarbital can inhibit surfactant synthesis by type II cells (25,26,27).

Since type II cells have been shown to respond to hormones and a number of drugs, the possibilities exist that these cells may respond in some manner to important toxins. Das (40) administered aflatoxin B₁ to gravid rats on day 17 and 19 of gestation. The fetuses responded with increased lung neutral lipid synthesis but a significant decrease in phosphatidylycholine synthesis — a major component in surfactant. The lamellar bodies appeared normal but there was significantly less surfactant secreted which the authors interpreted as an inhibition of the unfolding processes by which surfactant is secreted. Similar effects could be observed with colchicine and vinblastine, two microtubule inhibiting drugs (29) which would suggest that in type II cells aflatoxin B₁ can inhibit the mechanism of secretion possibly through inhibition of microtubule formation.

Of further interest in type II cells to toxicologists would be the ability of these cells to develop resistance to toxins. Initial exposure to sublethal concentrations of O₂ or Ozone (O₃) confers some resistance to experimental animals when they are exposed at a later time to higher concentrations of these toxic gasses (30). Hyperplasia of type II cells and alterations in the activity of numerous enzymes in homogenates of lung tissue accompany this resistance to injury. Delucía (31) and others (32,33) have demonstrated increases in glucose-6-phosphate dehydrogenase activity in lung homogenates after animal exposures to oxidant lung toxins such as 81% O₂ for 4 days. The
augmented G6PD activity probably reflects increased activity of the hexose monophosphate shunt, which in turn can be related to increased levels of NADPH required for reduction of oxidized glutathione. Other enzymes shown to increase in lung homogenates include 6-phospho-gluconate dehydrogenase and malic enzyme, glutathione reductase, and other disulfide reductases, and glutathione peroxidases (34).

While type II cells are mainly credited with secreting surfactant, they may also secrete other materials of a less defined function. A variety of lysosome enzymes exist in lamellar bodies as identified by cytochemical and biochemical studies (35). These enzymes are secreted along with the lipids of surface active material, but the functions of these enzymes are not known. It is possible that they have an important physiological function in clearing materials from the lung surface or in altering the functions of other lung cells. One particular enzyme found in the lamellar bodies in type II cells is phosphatidic acid phosphohydrolase. This enzyme is used as a marker in the amniotic fluid to determine the maturation of the fetal lung (36). As the lung develops, the levels of this enzyme increase.

The third cell type in the alveolar area, the capillary endothelial cells, are similar in appearance to type I cells but only 25% as large (37). The attenuated part of the cell is 0.1 to 0.2 μm thick. Normally the endothelial cells are arranged such that cell to cell junctions occur in zona occludens much like the epithelial cells. In some lung diseases, however, the junctions may be arranged in fenestrations. This is occasionally seen in alveolar fibrotic diseases (38).
The endothelial cells have been shown to possess intracellular filaments at the zona occludens which may act as a valve mechanism owing to their contractile nature (38). On the other hand contractile cells (Pericytes) are known to envelope alveolar capillaries and may regulate the regional blood flow to the gas exchange cells.

The endothelial surface is also corrugated by filiform projections and pits that represent vesicles open to the surface. The endothelial cells contain a great many vesicles which are rather uniform in diameter (50-80 nm). The openings of some of the vesicles into the lumen of the capillary appear to be capped by a diaphragm and may provide a means for movement of large molecules across the endothelium (39). On the concave surface of the vesicles have been identified globular structures of the order of 8.5 nm which may represent enzyme clusters particularly 5' nucleotidase and converting enzyme for bradykinin and an angiotensin I (40). Thus, it can be speculated that the endothelial cell vesicles may have two functions. One would be the transcapillary movement of lipid insoluble macromolecules through the vesicles and two, the metabolism of plasma constituents at the cell surface.

The fourth important cell type in the alveolar region resides on the epithelial surface of the lung. These cells, pulmonary alveolar macrophages (PAM), although they offer no structural contribution to the lung, nonetheless, do play an integral part in the defenses of the mammalian lung and are present in such great numbers (22.2-30.8 x 10⁶ cells/lung) that this discussion must include these cells (41). In our
own work with the lung, the PAM play an important role in the design and measure of success of our experiments.

Briefly, the PAM are phagocytic cells of the lung (41). They are derived mainly from the bone marrow (42). The direct precursor of the PAM appears to be an interstitial cell derived from circulating monocytes, which in turn, arise from a bone marrow precursor cell. The interstitial cell can proliferate, and it may be in the interstitium that the PAM develop the functional and metabolic features that distinguish it from the blood monocyte. Recently, it has been established that rodent PAM may proliferate in vivo and in vitro (43,44).

The size of the PAM may vary from 10-50 μm in diameter (45). The nucleus to cytoplasm ratio is variable but is commonly about 1:3. Nucleoli are often visible and multi-nucleated PAMs are frequently seen. Cytochemical studies indicate PAMs contain large quantities of nonspecific esterases (45). These cells stain positively with PAS staining techniques and possess very little lipid material. PAMs have been shown to exist on the surface lining of the alveolar surface of the lung (46). PAMs contain a well-developed golgi apparatus as well as some mitochondria (45). The endoplasmic reticulum is not extensive and is predominately rough. Free ribosomes and glycogen granules are often present.

The layer between the epithelium and the endothelium comprises the alveolar-capillary interstitium. The interstitium consists mostly of basement membrane from the endothelium fused with that of the type I and II cells (47). In regions other than the alveolus, the basement membrane widens to enclose cellular elements such as fibroblasts,
pericytes, collagen and elastic fibers as well as mast cells (48). The interstitium is responsible for the structural stability of the lung. The tough collagen-elastic makeup of the basement membrane allows stretchability and structural support as well as the permeability to gases necessary for ventilation. For mammalian lungs it can be said that on the average endothelial and epithelial cells each contribute 38% to the total thickness of the barrier with the remaining 40% due to interstitium (7).

Upon the surface of the alveolar epithelium there exists a surfactant layer. The composition of the surfactant layer has only recently been studied (49,50,51). Surfactant layer is a bilayer consisting of a surface layer of saturated phospholipids predominantly dipalmitoyl lecithin and a basal layer containing proteins, polysaccharides and phospholipids (51). The layer of surfactant is of varying thickness but averages 50 nm which offers very little impedance to gaseous diffusion (50). Surfactant promotes alveolar stability at varying degrees of pulmonary distension, endows the lungs with many of its elastic properties and protects the lungs against edema by preserving the proper hydrostatic pressures across the alveolar-capillary membranes (49). The regulation of surfactant is accomplished via type II cells which secrete surfactant and alveolar macrophages which play an important part in the removal of surfactant (52). The relationship of the two processes is influenced by lipid metabolism and hormones (thyroxine, corticoids) and may be further influenced by acute hyperventilation (53).
Alterations in any of the processes and functions of the lung which were just described may provide the mechanism for an observed toxic response in the lung and ultimately the entire animal. Alterations in surfactant production, for instance, either through increased or decreased production by type II cells or increased or decreased clearance by macrophages and other clearance mechanisms, may affect not only the site of change, but may influence the environment of all cells in the lung because of the protective role surfactant plays. For example, respiratory distress syndrome in newborns is characterized by insufficient surfactant productions leading to compromised gas exchange (54). This effect eventually is life-threatening to the infant. The initial toxic effect is exerted biochemically on a developing functional aspect of one of the many cell lines within the lung (type II cells). This one functional alteration in turn affects more widespread processes in the lung eventually leading to a response by the entire organism.

In addition to mechanisms directly affecting function, toxic responses also may be due to alterations initially in structure. Because lung function so closely follows structure, alterations in structure may very likely lead to impairment of normal functions of the lung, again leading to a toxic response.

A number of toxic agents act in this manner. BHT, bleomycin, nickle, silica, coal dust, coal fly ash, 80% O_2 4 days, O_3, NO_x, paraquat and diaquat all exert their primary toxic effects through changes in the structure of gas exchange regions (55) through increased
collagen synthesis and thickening of basement membrane. This change in structure results in an increase in gas diffusion path length in the alveolus, thereby decreasing the $D_x$ value in Fick's Law and impairing gas exchange.

The changes in structure caused by chronic exposure or acute administration of these agents not only affects the $D_x$ term in Fick's Law by increasing pathlength but also by changing the solubility function of the term (5). Solubility of gases through collagen and other fibrotic materials is different from that of solubility in the type I gas exchange cells leading to alteration in diffusion.

Structurally these components may impair the mechanical aspects of respiration by decreasing compliance of the lung (55,56). Lambertson and Clarke (57) reported 20-30% decrease in lung compliance in rats chronically exposed to 60% or greater levels of $O_2$ or to paraquat 25 mg/kg (58). In these situations, the entire organism is under increased stress since more effort is required for normal respiration thereby placing the animal in a higher risk state for response to other toxins or diseases.

There is yet another function which occurs in the lung that is commonly affected by pulmonary toxins. The endothelial cells, basement membranes, epithelial cells, lymphatics and surfactant layer cell function is a coordinated manner to maintain a proper fluid balance in the lung. Since the blood and thin alveolar barrier are in such intimate contact, there is ample opportunity for fluid infiltration to the critical alveolar region. Toxins which affect this important
regulation in the lung will cause pulmonary edema and further threaten
the entire animal. Paraquat and oxygen are two toxins which have been
studied extensively and which exhibit increased pulmonary edema as a
result of insult. In lung response to paraquat and $O_2$ toxicity, the
overall scheme of response is generally consistent and reproducible.
During chronic exposure to 80% $O_2$ or paraquat insult, there is an
initial phase of response characterized by inflammation, edema,
hemorrhaging, swelling and destruction of alveolar type I cells. These
changes were most evident after 4 days continuous exposure to 80-100%
$O_2$ or a single 25 mg/kg i.p. injection of paraquat dichloride (57,58).
Oxygen is thought to initiate these responses due to oxidation of
critical membrane structures (57). Ultimately, paraquat is thought to
act in this manner also but through a more oblique approach.

The initial biochemical events that lead to the paraquat-induced
lung lesions are not precisely defined; however, Bus et al., Fisher
et al., and Baldwin et al (59,60,61) have provided evidence suggesting
that paraquat induces lung lesions initially through an increase in
lipid peroxidation of cellular membranes. Paraquat can undergo a
cyclic NADPH-dependent reduction, $O_2$-mediated reoxidation. This
reoxidation results in production of singlet oxygen which interacts with
the polyunsaturated fatty acids to produce lipid peroxides. These
peroxides in turn produce lipid free radicals which interact with
additional unsaturated fatty acids in the cell membranes. Unless there
is a sufficient concentration of free radical scavengers or antioxidants
available to quench this autocatalytic self-sustaining reaction, tissue
damage and necrosis will occur.

It is speculated that the type I cells are most susceptible to
this type of damage because of their flat, delicate structure (58).
This structure, as was discussed before, is necessary to offer the
shortest path length possible for gaseous diffusion. This also means,
however, that they possess very little cytoplasmic volume and a large
surface area of cell membrane. The extensive cell membrane surface
area offers ample sites for lipid peroxidation so each type I cell has
a higher risk of damage from these agents. As the type I cells are
damaged or destroyed the structure of the alveolus is altered. Type II
cells, basement membrane, and endothelial cells are left. The fluid
regulatory properties of the type I cells are missing and an invasive
edema is observed.

The processes described above are usually only the initial
toxic effects. In most cases, if the animal survives the initial phases
of edema, there is very often a chronic or proliferative phase which
ensues. It is during this phase that type II cell proliferations
occur, interstitial fibrosis, macrophage recruitment and fibroblast
appearances are noted (57,58).

If the initial insult is severe enough, the basement membrane
can likely be affected. Without the normal scaffolding of the basement
membrane to direct repopulation of the alveolus by differentiating type
II cells, fibrosis will ensue. With fibrosis, structures of the
alveolar regions are altered. Diffusion path length is increased and
solubility of gases through the alveolar barrier is changed. Hence, alteration of fluid regulation in the lung results in a sequel of events which ultimately leads to impaired gas exchange which in turn leads to systemic involvement in the toxic response.

It is interesting to note the role type II cells have in response of the lung to oxidative type insults such as those just described. The type II cells have the responsibility of repopulating the alveolus after damage to type I cells. Once the alveolar region is resurfaced with type II cells, differentiation of these cells occurs and type I cell morphology is observed. Why type II cells undergo differentiation or how, or by what mechanism are type II cells more resistant to oxidative damage have yet to be answered.

Further interest in these cells has been generated by evidence that type II cells isolated from rabbit lung also possess some drug metabolic capabilities. Devereux et al. (62) showed inducible P-450 and P-448 activity in isolated type II cells and further showed that cell fractions enriched in type II cells possessed 7-ethoxycoumarin de-ethylase activity as well as coumarin hydroxylase activity. Work by Roberts et al. (63) has shown the $O_2$ injury to lungs of rabbits can be altered by the use of some pharmacologically active compounds. Lungs from animals treated with dexamethasone (0.4 mg/Kg/day) were found to have greater $O_2$-induced lung damage and significantly lower pulmonary antioxidant activity. Rats pretreated with propylthiouracil (10 mg/Kg/day ip) showed less $O_2$ damage with greater pulmonary reduced glutathione levels and catalase activity in type II cells. Therefore, it has been
shown that type II cells can respond to pharmacologic or toxicologic agents either through cytochrome P-450-448 activities or through non-microsomal processes.

Because of these functions and those mentioned earlier in this essay, it was decided that type II cells would be of great interest to toxicologists. Any one of the number of normal functions and capabilities of the type II cells, such as surfactant production, could be investigated as an index of toxicity within the lung. Their role in the lung's response to $O_2$ toxicity or paraquat would suggest that type II cells were of interest to toxicologists wishing to investigate the important mechanisms of resistance and differentiation.

To adequately study these functions of type II cells it was decided that isolated preparations of cells should be obtained. Although it would be more appropriate to study the functions of type II cells in situ, the technical difficulties involved in such a study would prove to be enormous. Studying isolated cells would offer the advantage of a controlled environment for the cells without the influences from neuronal or hormonal systems. It also would mean that intracellular events could be measured and attributed to the cell in question without overt concern that effects observed were due to other cell lines in the preparation. There would be the further advantage of obtaining replicate samples within each experiment so animal to animal variation could be minimized.

A review of the literature has shown a number of methods available at present for isolating type II cells in a viable state from
lung fractions of rabbits and rats. These methods generally involve disruption of the organ through the use of proteases, then centrifugation through a discontinuous gradient. In our hands these methods all required approximately six hours of preparation before cells could be obtained in sufficient numbers to conduct experiments. A method was needed which would allow reproducible isolation of cells in high enough purity to allow us to conduct experiments before cellular alteration occurred.

The initial work in isolating viable type II cells followed the procedure of Kikkawa and Yoneda (64). With the original procedure, elements of blood were eliminated by simple manual perfusion with Joklik solution through the inferior vena cava. Removal of the free lung cells was accomplished by alveolar washing via the trachea, dissection of soft pulmonary tissue from the airways, and mincing the lung tissue and mechanically shaking the minced lung suspension. This step removed 75% of the PAMs. Subsequent trypsination for 20 min at 37°C detached almost all of the epithelial cells leaving intact interstitium and capillaries. The yield at this step was approximately 10 million cells/rat lung of which 30% were type II alveolar epithelial cells. Cell suspensions were then treated with Barium sulfate or Baraloid. The Barium particles were engulfed by the macrophages offering a greater density than normal to the macrophages. Normally, macrophages and type II cells have a similar weight and density and therefore separate upon the same layers with discontinuous bouyant centrifugation. Presumably after the Baraloid treatment the macrophages were considerably heavier. The cell suspension containing type II cells and Baraloid
laden macrophages were layered on discontinuous ficoll gradient, densities 1.047 and 1.058, and centrifuged for 1 hour at 1700 × g. The zone above the 1.047 density ficoll gradient contains 6% of the original number of cells in suspension and allegedly consisted of approximately 90% epithelial type II cells. The cells were then removed from the zone layer above 1.047 g/ml washed in fresh buffer and checked for viability. With the procedure, Kikkawa and Yoneda were able to isolate suspensions of epithelial type II cells of 95% purity.

When we attempted to duplicate Kikkawa's work, a number of problems arose. Cell viability fell below 80% and cell yield was less than 2 × 10⁶ cells per rat. Most importantly, cell purity was far below what Kikkawa reported. In a personal communication with Dr. Theodora Devereux of NIH (65) we learned that others have also failed in their efforts to obtain reasonable yields of high purity.

We then investigated another procedure for isolating viable type II cells. This method was developed by Dr. Mason and his associates at the University of California at San Francisco (66). In this method the isolated perfused lungs were instilled directly with a fluorocarbon-albumin emulsion then incubated at 37°C in a water bath for 20 min. The lungs were then instilled with trypsin 0.3% and after 20 min. the lungs were minced and filtered. A soybean trypsin inhibitor was added to the cells and the suspension shaken in a 250-ml Erlenmeyer flask. The minces and free cells were then decanted through a series of filters. The cells were then layered over a discontinuous gradient composed of 10 ml of 1.040 density albumin with 10 ml of 1.080 density albumin
solutions. Centrifugation proceeded at 315 x g for 20 min. Purity was reported to be 60% type II cells with a yield of 20.3 x 10^6 cells per rat.

When conducting these experiments we encountered a number of difficulties in design and application. As with the method Kikkawa, the apparent yield of the cells from rats was significantly less in our attempts than reported by Mason and his group. In their findings 8.3 x 10^6 cells per animal at 68% purity was the average yield, while our experiments yielded 1 x 10^6 cells per animal on the average or less. In their discussion, Mason and his group addressed the problem of using animals that were suffering from respiratory diseases. They were able to demonstrate that cell yield and viability of cells obtained from sick animals were significantly less than from pathogen-free or even healthy, normal rats. They further discussed the tremendous variability in results that were achieved not only from day to day but from experiment to experiment within the day. This variability was adequately demonstrated by our laboratory during experiments we conducted with this method. We obtained yields varying from 10 x 10^6 to 5 x 10^5 cells after purification and with purities ranging from 38% to 70%. Viability in all our preparations was low -- often less than 65%.

For much the same reasons that we found Kikkawa's method unacceptable, we also found Mason's method unacceptable.

More recently a method developed by Dévereux et al. (62) at NIH separated cells dispersed from the lung on the basis of their size in a method known as centrifugal elutriation. In this system cells are
placed in a chamber in the elutriator rotor where centrifugal forces pellet the cells to the outside of the chamber. A counter-current flow of buffer moves the cells of the pellet into a collecting vial based on considerations of cell size, angular velocity of the rotor, and flow rate of the buffer. In this way Devereux et al. were able to enrich fractions in type II cells to approximately 40% ± 15% SE. This enrichment is by no means to be considered a pure preparation, but it should be appreciated that there is an enormous time savings achieved in this method and there is increased viability of the cells obtained (greater than 90%).

Thanks to the generosity of Dr. Eugene Gerner of the Department of Radiation Oncology at The University of Arizona, we were able to use an elutriator system to attempt to isolate these cells. We were able to duplicate the results of Devereux quite consistently. We further improved the purity of the type II fractions obtained by this method. Devereux’s method was a combination of altering the speed of the rotor head and the flow rate of the buffer to affect elutriation of the fraction from the pellet. When increasing or decreasing the angular velocity of the rotor, the time required for the head to speed up or slow down can alter the purity of the preparation obtained. As the rotor is in the transitory phase from one speed to the next, there exists a period where forces counteracting one another (centrifugal and countercurrent forces) are in a state of flux and separation during this time becomes imprecise. In our method we were able to show very clean and precise separation by changing the countercurrent flow rate (which can be accomplished at defined increments) while leaving the angular
velocity of the rotor constant. We obtained type II cell concentrations in excess of 60% ± 10% SE utilizing this altered technique with viabilities of greater than 90% ± 2% SE. The most encouraging part of this preparation is the time savings over the other methods. We were able to complete these experiments in less than 3 hours. Recently a paper by Mason (67) used a combination of the elutriator separation with ficoll gradients to offer a separation in excess of 80% type II cells.

There are some considerable difficulties in this elutriator system. Primarily there is the consideration of cell purity, 60% enrichment of type II cells as we obtain from this system is good, but with this method of preparation there are two major contaminating cell types, macrophages (45) and clara cells (68). This latter cell type is reported to be high in drug metabolic activity.

We are also still confronted with the problem of poor cell yields via this preparation. Devereux reports obtaining 12 x 10^8 cells from the whole cell dispersion of rabbit lungs and recovering 81 x 10^6 cells in the type II fractions. We routinely obtained less than 5 x 10^6 cells in any one fraction in the elutriator, which is still significantly greater than we have been able to obtain by any other method. In order to meet our needs, the cell yields, purity and viability had to be improved.

All of the methods reported so far attempt separation of cells based on density or size differences between cells. As we mentioned, type II cells and macrophages can be quite similar in both size and density. Because of this, separation of cells based on these parameters will offer incomplete separation of cells.
The use of cultured cells represents another approach to producing viable type II cells. Reports, initially by Douglas et al. (69), then later by other groups (70,71,72), have claimed to be able to produce type II cells in culture. However, recent reviews of these techniques have revealed that the cells in question do not possess many of the qualities of cells in the intact lung (73). Surfactant synthesis was shown to be altered and morphologic evaluation of the cells showed decreased number of characteristic inclusion bodies. A number of groups (74,75) have reported that type II populations are unstable under culture conditions which makes claims of successful cell cultures suspect. We conclude from the available literature that, at this time, culture techniques to isolate type II cells were too uncertain and cells obtained from such methods were of questionable identity. Results from our preliminary efforts to duplicate published methods to isolate type II cells are shown in Table I.

**Statement of Purpose**

It was the objective of this research to develop a rapid, effective method for isolating viable type II alveolar cells which could be utilized in primary culture experiments to assess toxicological or pharmacological properties of compounds on these cells. In procedures reported previously, size or density differences have been utilized to separate cell lines from the lung.
Table I. Comparison of Methods for Isolating Type II Cells. — No statistics were conducted. In our hands most literature values could not be duplicated. However each method was only conducted four times.

<table>
<thead>
<tr>
<th>Method</th>
<th>Cell Yield After Enzymatic Dispersion Cells/Lung</th>
<th>% Viability</th>
<th>Cell Yield After Purification</th>
<th>% Type II Viability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lit.</td>
<td>Ours</td>
<td>Lit.</td>
<td>Ours</td>
</tr>
<tr>
<td>Kikkawa (64) Rat Lungs</td>
<td>9x10^6 ±1x10^5</td>
<td>1x10^6 ±3x10^5</td>
<td>88</td>
<td>55</td>
</tr>
<tr>
<td>n=40</td>
<td></td>
<td></td>
<td>±11</td>
<td>±20</td>
</tr>
<tr>
<td>Mason (66) Rat Lungs</td>
<td>30x10^6 ±5.1x10^6</td>
<td>3x10^6 ±1x10^6</td>
<td>90</td>
<td>78</td>
</tr>
<tr>
<td>n=25</td>
<td></td>
<td></td>
<td>±2</td>
<td>±5</td>
</tr>
<tr>
<td>Devereux (62) Rabbit Lungs</td>
<td>400x10^6 ±8x10^6</td>
<td>30x10^6 ±3x10^6</td>
<td>91</td>
<td>90</td>
</tr>
<tr>
<td>n=8</td>
<td></td>
<td></td>
<td>±3</td>
<td>±6</td>
</tr>
<tr>
<td>Greenleaf (67) Rat Lungs</td>
<td>34x10^6 ±8x10^6</td>
<td>11x10^6 ±6x10^6</td>
<td>90</td>
<td>88</td>
</tr>
<tr>
<td>n=10</td>
<td></td>
<td></td>
<td>±5</td>
<td>±6</td>
</tr>
</tbody>
</table>
Our approach has been to remove macrophages, the major contaminant of type II preparation, based not on size or density but based on the phagocytic characteristics. Other methods have utilized the phagocytic nature of PAMs to load them with a dense material such as BaSO₄ or fluorocarbon to improve density separation. We reasoned that if we could create a suspension of magnetic iron oxide particles we could introduce them into the macrophages through phagocytosis then remove these magnetic cells from a preparation of pneumocytes with a strong magnetic field.

This device has been used in the past to isolate intact viable glomeruli from rabbit kidneys (76). A suspension of magnetite was perfused through the renal artery and the magnetite particles filtered into the glomeruli. After disruption of the kidney by gentle homogenization, the intact glomeruli were pulled away from the rest of the preparation by placing a strong permanent magnet to the preparation.

A report by Senyei et al. (77) described the physical limitations of removing magnetic bodies from a model flow system. This paper provided information on optimal flow speeds required for removing magnetic spheres as well as magnetic strengths required and distances for maximal magnetic influence.

To further develop this method for isolating type II cells it was necessary to evaluate the effectiveness of proteolytic enzymes in removing alveolar cells from their basement membrane. Most published methods utilize trypsin for two reasons. Primarily it is an effective enzyme for releasing cells from the matrix in the lung. Secondly, it
appears that trypsin in concentrations 0.1 to 1% can preferentially release type II cells from their basement membrane (78). This property of trypsin makes it particularly useful for isolating type II cells since the preferential release of type II cells represents an enrichment procedure of its own regardless of the enrichment steps which follow. There exists a major disadvantage to using trypsin which prompted us to evaluate other proteolytic enzymes for type II cell preparations. Trypsin is effective in removing cells from the basement membrane but since it is rather unspecific in its proteolytic activities many cell surface functions are destroyed and as a result viability is low in cells treated with trypsin. Further, trypsin treated cells tend to clump, possibly because of altered surface proteins. This clumping of the cells made separation of cells from the clump very difficult and reduced yields in the remaining steps. Finkelstein and Mavis (39) reported that hepatocytes treated with trypsin showed reduced membrane cytochrome b$_5$ activity compared to cells not treated with trypsin. Since many of the toxicological or pharmacological evaluations may rely on unaltered membrane receptors we decided another proteolytic enzyme may offer advantages over trypsin.

In addition to considerations of which enzymes to use, the method of introducing the enzymes to the lung became important to the development of the isolation procedure. Introduction of enzymes to minced lung preparations released a different profile of cell types compared to direct instillation into the trachea. Therefore, the proper method of introduction of enzymes became important to the method development.
Once the method was developed for isolating type II pneumocytes, cell metabolic characteristics would be investigated to determine the suitability of the cell preparation as a toxicological or pharmacological tool.
MATERIALS AND METHODS

Animals

White New Zealand male rabbits (2-3 Kg) were obtained from a local vendor (Blue Ribbon Ranch). Rabbits were fed standard Purina® Chow ad lib and maintained in a temperature controlled room (22° C) with 12 hrs of light and 12 hrs of darkness in the University of Arizona Division of Animal Resources facilities.

Buffers

A HEPES buffer was prepared as the perfusion medium containing 128 mM NaCl, 2.5 mM sodium phosphate buffer, 17 mM HEPES, 5.5 mM glucose, 5 mM K₂SO₄ and 5 mM KCl and 10% fetal calf serum. All solutions were prepared no more than 24 hrs in advance, millipore filtered and oxygenated with 95% O₂, 5% CO₂, 20 min prior to use. Buffers were adjusted to pH 7.4 with 2 N NaOH.

CaCl₂ and MgCl₂ Solutions

To prepare these stock solutions 2.79 g CaCl₂·2H₂O and 2.53 g MgCl₂ were added to 100 ml distilled water and stirred until dissolved. For the dilute working solution 2.5 ml were added to 100 ml of the appropriate buffer.
EGTA Solutions

190 mg EGTA [ethyleneglycol-bis-(β-amino-ethyl ether) N,N'tetraacetic acid] were dissolved in 10 ml distilled H₂O. Three drops of 10 N NaOH were added to raise the pH to 9.0. One ml of this stock solution added to 100 ml perfusion buffer resulted in a final concentration of 0.5 mM EGTA.

EDTA Solution

2.08 g EDTA (ethylene diamine tetraacetic acid-tetra sodium salt) was added to 10 ml of distilled water with 2 drops 10 N NaOH; 1 ml of stock solution was added to 100 ml of buffer resulting in a final concentration of 0.5 mM EDTA.

Magnetite Preparation (Fe₃O₄)

Magnetic iron oxide (magnetite) was prepared freshly for each experiment. Two ml of 250 mM FeSO₄ and 2 ml 250 mM FeCl₃ were added dropwise to 50 ml of boiling 0.2 N NaOH. Precipitate formed immediately and the mixture was allowed to boil 10 min. The precipitate was removed from the beaker by decanting the liquid phase then resuspending the precipitate in 10 ml of distilled water. The suspension was transferred to a 50 ml centrifuge tube and centrifuged at 100 x g for 5 min. The supernatant was removed by aspiration and the precipitate washed three additional times in the same manner. The washed magnetite was then suspended in 10 ml of fetal calf serum (GIBCO) and dialyzed overnight against distilled water. The dialysate was sonicated under cooling at 50% pulsation mode -- 50%
maximal output with a Sonifier model 350 (Branson Sonic Power Co.). The preparation was transferred to a 20 ml glass vial and kept at \(-20^\circ C\) until used.

NOTE: The colloidal iron oxide was not allowed to come into contact with strong magnetic fields at any time as this would cause the particles to magnetize and aggregate the suspension.

**Trypsin Solutions**

Two grams crude trypsin (Sigma) were dissolved in 20 ml of HEPES buffer and stored at \(-20^\circ C\). From this 0.1 g/ml (10%) solution appropriate dilutions were made with HEPES buffer to yield 1.0, 0.75, 0.5, 0.25 and 0.1% trypsin solutions for experiments outlined in the methods section.

**Trypsin Inhibitor DNAase Solutions**

The solution with trypsin inhibitor was made freshly for each experiment by dissolving 1 mg of soybean trypsin inhibitor (Sigma) and 30 \(\mu \text{g}\) of DNAase (Sigma) per ml of buffer. Routinely a 250 ml volume was made for each experiment; this required 250 mg trypsin inhibitor and 7.5 mg DNAase.

**Thermolysin Solutions**

Stock solution was prepared by adding 100 mg thermolysin (Sigma) to 100 ml HEPES buffer and stored at \(-20^\circ C\). From this 1 mg/ml solution appropriate dilutions were made to yield 10, 20 and 30 \(\mu \text{g/ml}\) solutions for experiments outlined in the methods section.
Collagenase Solutions

Fifty mg collagenase (type CLS II Worthington Biochemical) were added freshly to 10.0 ml HEPES buffer. From this 1% solution appropriate dilutions were made with HEPES buffer to yield 0.025, 0.05 and 0.1% collagenase solutions for experiments outlined in the methods section.

Elastase Solutions

Elastase (11 mg protein/ml Sigma) was added freshly to HEPES buffer to produce 2.2, 1.1 or 0.55 mg% elastase solutions as outlined in the methods section.

Pronase Solution

One g pronase (Sigma - B500S) was dissolved in 10 ml of HEPES buffer and stored at -20°C. From this 10% solution appropriate dilutions were made with HEPES buffer to yield 0.5, 0.25, 0.1 and 0.05% solutions of pronase for experiments outlined in the methods section.

Karnovsky's Fixative

Sodium Cacodylate buffer was prepared by adding 7.99 g Na Cacodylate (Sigma) to 250 ml distilled water.

To prepare Karnofsky's solution for fixing the cells, 2 g of paraformaldehyde (Eastman) was dissolved in 25 ml distilled water. The solution was cleared by the addition of 2-3 drops of 1 N NaOH. The mixture was then allowed to cool to room temperature and 10 ml of 25% glutaraldehyde (Baker Chemical Co.) were added to the solution. The
preparation was then brought to 50 ml with cacodylate buffer. The pH was adjusted to 7.2 with 2-3 drops 2 N HCl. Following the addition of 32 mg CaCl₂·2H₂O (MCB Chemical Co.) the solution was diluted 1:1 with cacodylate buffer.

**Trypan Blue Stain**
Commercially prepared trypan blue was used to test for cell viability. 0.4% trypan blue stain (GIBCO #525) in normal saline was diluted 1:3 with buffer to yield a 0.1% solution for viability tests.

**Reagents for Papanicolaou Stain**

**Harris's Hematoxylin**
2.5 g Hematoxylin (Eastman) was dissolved in 25 ml absolute ethanol (U.S. Industrial Chemical Co.). Aluminum ammonium sulfate (Fisher Scientific Co.) was dissolved in 50 ml distilled water. The two solutions were mixed slowly and brought to boiling. The solution was removed from heat and 1.25 g Mercuric oxide (Mallinckrodt) was slowly added. The solution was reheated until it became dark purple. It was then cooled rapidly in an ice bath and filtered through a Whatman #1 filter before use.

**Lithium Carbonate**
A saturated solution of lithium carbonate (Mallinckrodt) was prepared in distilled water. To use, a 1:100 dilution with distilled water was conducted.
Orange G and EA-50

Orange G and EA-50 were obtained commercially from Harleco Biological Stains and were used undiluted.

Ethanol Washes

50%, 80%, 95% absolute ethanol solutions were made with distilled water and absolute ethanol (U.S. Industrial Chemical Co.).

Xylene Ethanol Wash

Xylene (U.S. Industrial Chemical Co.) and absolute ethanol (U.S. Industrial Chemical Co.) were mixed in a 1:1 ratio.

Acridine Orange Fluorescent Stain

For fluorescent staining, acridine orange was used (1 μg/ml). A stock solution of 100 μg/ml was prepared by adding 10 mg acridine orange (Fisher) to 100 ml of distilled water. To use, 0.1 ml of stock solution was added to 9.9 ml of HEPES buffer.

Osmium Tetroxide Solution for Lipid Reaction

Osmium tetroxide (Electron Microscopy Sciences) was dissolved in carbon tetrachloride (100 mg/ml) and stored in the dark at 4°C until used. From this stock solution, a working solution of 6.2 mg/ml was made by adding 6.2 ml of stock to 93.8 ml of carbon tetrachloride.

\[ O_2 \] Measuring Equipment

Experiments to determine the oxygen consumption rates of isolated cells were conducted with a Clark type oxygen electrode. The electrode used in these experiments had a 1.9 ml cell chamber and a
polypropylene electrode membrane obtained from Instrument Laboratories Inc. A Linear Instrument recorder model 252A was used to monitor the signal changes from the electrode.

**Preparation of Lung**

Animals were anesthetized with 50 mg/Kg sodium pentobarbital and treated with 2500 units/Kg sodium heparin by a single intraperitoneal injection. Fifteen min later, the ventral side of the animal was incised and the aorta transected. Both the trachea and the pulmonary artery were cannulated with 18 ga. steel cannulas. After the cannulas were in place, the heart was trimmed away from pulmonary tissue. The pulmonary artery cannula was attached to an elevated 3 l aspirator bottle containing perfusion medium and the lungs perfused.

Additionally during perfusion, 20 ml of fresh buffer were instilled into the lung via the tracheal cannula to remove free macrophages. After 1 min, this buffer was removed and replaced with 25 ml of fresh buffer. The tracheal cannula was then closed with hemostats. The second instillation of buffer remained in the lung until after vascular perfusion.

Thus prepared, the lung system was removed en bloc and attached to a continuous perfusion apparatus through the pulmonary artery cannula. Warmed and oxygenated buffer was perfused continuously through the lung in a recirculatory manner at a flow rate of 35 ml/min (see Fig. 1).

Once continuous perfusion began, the hemostats were removed from the trachea and the intra-tracheal lavage fluid removed. With a
Figure 1. Recirculatory Perfusion Apparatus.

Lungs are removed *in bloc* from the rabbit and the pulmonary artery cannulated. Perfusion is accomplished through pulmonary artery in a recirculatory manner with warmed and oxygenated buffer.
25 ml syringe, 20 ml colloidal iron oxide were instilled into the lungs via the tracheal cannula. The perfusion buffer was replaced at this time with 100 ml fresh buffer supplemented with 2.5 ml of 5.0 mM CaCl₂, MgCl₂ solution. After 30 min, the used perfusion buffer in perfusion apparatus reservoir was replaced with fresh buffer containing no CaCl₂ and MgCl₂ supplement. The lung was lavaged through the tracheal cannula three additional times with 20 ml of this buffer. Prepared lungs were then ready for enzymatic dispersion.

**Enzymatic Dispersion of Lung Matrix**

Enzyme solutions were instilled into the lungs via the cannulated trachea. After instillation of enzymes, the trachea was closed with hemostats to prevent material leaking back through the cannula. At this time, the perfusion buffer in the perfusion apparatus reservoir was replaced with fresh buffer containing enzyme and lung perfusion continued through the pulmonary artery. In this way, proteolytic enzymes were instilled into alveolar regions via tracheal instillation and enzyme was introduced to the endothelial side through perfusion medium via pulmonary artery. Usually after 10 min the lungs collapsed, at which time they were filled through the trachea with additional enzyme solution and incubated for an additional 10 minutes. During this last 10 minutes the lungs became edematous to the point where fresh enzyme and buffer often were added to keep buffer levels in the perfusion apparatus at proper heights.
This completed the first portion of the experiment which remained constant regardless of which of the following two methods were used for harvesting cells.

Removal of Cells, Method 1

After enzymatic digestion, 100 ml of perfusate was removed through the trachea in 10 ml steps. To avoid depletion of buffer, 10 ml of fresh buffer were added to the perfusion apparatus reservoir when each 10 ml was removed from the lungs. After removing each sample, the lungs were gently massaged to free more cells. The material recovered in this way contained alveolar epithelial cells. After the fluid was centrifuged at 50 x g for 10 min, the pellet was resuspended in 2 ml fresh HEPES buffer supplemented with EGTA. This constituted method #1.

Removal of Cells, Method 2

The lung was removed from the perfusion apparatus, without removing perfusate and cells from the trachea, and placed on a 15 cm siliconized watch glass. Both cannulas were removed after the trachea and visible hilar structures were cut away, the lungs were minced with sharp scissors until the pieces were no larger than 0.5 cm. The pieces and fluid from mincing were transferred to a 250 ml siliconized Erlenmeyer flask and brought to a 200 ml volume with HEPES buffer supplemented with either 0.5 mM EGTA or 0.5 mM EDTA. The flasks were shaken for 10 min in a gyrotry shaker bath (New Brunswick model G-76) at a setting of 8 (approximately 100 rotations/min). The temperature was maintained at 37°C. Minces and free cells were decanted through
180-μm, 100-μm and 20-μm nylon mesh screens into centrifuge tubes. The filtrate was centrifuged at 50 x g (660 RPM on Sorval GLC-2) and the pellet resuspended in 2-ml fresh buffer. After centrifuging, gentle vortexing of the pellet helped resuspend the cells in a monodispersed pattern. This preparation constituted Method #2.

**Magnetic Removal of Macrophages**

Final steps in purification for both preparations 1 and 2 involved passing the cells through a strong magnetic field. To do this, a chamber was constructed with a narrow (2 mm) magnetic gap. Cells flowed through the chamber at a rate of 0.10 ml/min with a linear velocity of 2 mm/sec (See Figures 2 and 3). Effluent from the chamber contained a population of cells enriched in granular type II pneumocytes. Cells thus collected were washed one time in buffer, centrifuged at 100 x g, then resuspended in 4 ml fresh buffer.

**Cell Counting**

To count cells, 100 μl of cell suspension was placed in a microfuge tube containing 33 μl of 0.4% trypan blue solution and allowed to stand 1 minute before being counted on a standard hemocytometer. Cells in the middle 25 grids of the hemocytometer were used in the following calculation:
Figure 2. Separation Chamber.

Enlarged drawing of separation chamber. Note the baffles to reduce turbulene buffer flow. Note also the narrow magnetic gap <1 mm through which the cells pass.
A. Buffer Reservoir
B. Pump—Flow Rate
0.884 ml/min
C. Cell Suspension
D. 3-way Stopcock
E. Loading Chamber

F. Silastic Tubing
G. Separating Chamber
H. Funnel
I. Collecting Tube

Figure 3. Cell Separator.

Cell separation system allows the gentle movement of cells past the magnetic cap in the separation chamber.
\[ X = \left( \frac{Y}{1 \times 10^{-4} \text{ mm}^3} \right) \times \frac{133}{100} \times (Z) \]

\( X = \text{number of cells in solution} \)
\( Y = \text{number of cells counted on middle grids on hemocytometer} \)
\( Z = \text{volume of solution cells were obtained from} \)

Cell Viability — Trypan Blue Exclusion

Cell viability was determined, in part, during the cell counting procedure. If cells failed to exclude trypan blue, they appeared dark blue and were considered non-viable and counted as such. Lightly blue to colorless cells were counted as normal healthy cells. Viability was further determined through \( O_2 \) consumption and uptake of \( ^{14}C \) labeled acetate and \( ^{14}C \) choline.

**\( O_2 \) Uptake**

To determine cellular \( O_2 \) uptake, \( 10 \times 10^6 \) cells in 3 ml of fresh buffer with 200 \( \lambda \) fetal calf serum (GIBCO 250-6140) were used. This suspension was placed in the Clark oxygen electrode described in the materials section. After one minute equilibration, uptake of oxygen was monitored by measuring a decrease in partial pressure of oxygen (\( pO_2 \)) in the medium in 2 to 4 minute intervals.

Damage to cellular membranes was tested by adding succinic acid to the cell suspension in the \( O_2 \) electrode and monitoring for increased \( O_2 \) uptake. Electrode chamber levels of Na succinate were brought to 10 mM by adding 5.13 mg Na succinate (Calbiochem 5730, mwt 270.2). An
increase in O\textsubscript{2} uptake of more than 10% of basal levels was interpreted to mean some membrane damage had occurred and cells were no longer viable.

\textbf{\textsuperscript{14}C Acetate and \textsuperscript{14}C Choline Uptake}

Further tests of metabolic activity of isolated type II cells were conducted by lipogenesis experiments from radio labeled precursors. \textsuperscript{14}C acetate 5 \textmu mols (8 mCi/mmole) and \textsuperscript{14}C choline chloride 5 \textmu mols (21 mCi/mmole) obtained from New England Nuclear were added to 1 ml aliquots of cells (10 \times 10^6 cells). After incubation all suspensions were centrifuged at 1000 \texttimes g for 5 minutes. The supernatant was decanted and the cells osmotically lysed with 0.5 ml of distilled water. Lipids were extracted by adding 9.5 ml of chloroform/methanol (2/1 v/v) to lysed cells and shaking for 20 minutes. Two ml of 0.05 M KCl in distilled water were added and the 2 phases separated. The lower phase was evaporated to dryness.

The lipids were then reconstituted with 4 ml of carbon tetrachloride/methanol (2:1). This preparation was then washed with 0.8 ml 0.05 M aqueous KCl. The upper layer was removed and lower layer washed 3 times with 0.8 ml pure solvent upper phase CHCl\textsubscript{3}/MeOH/H\textsubscript{2}O (8/4/3) according to Folch et al. (80). The lower phase was then evaporated to dryness.

The lipid residue was reconstituted in 0.5 ml of osium tetroxide working solution and reacted according to the methods of Mason et al. (81). After allowing 15 minutes for completion of the reaction, the solution was evaporated and the residue was redissolved in
in chloroform:methanol (20:1 v/v). This material was applied to a column formed by placing 0.8 g of aluminum oxide (neutral alumina 100-200 mesh, Biorad Laboratories) on a plug of glass wool in the neck of a disposable 9" Pasteur pipette.

Neutral lipids not altered by the osmium tetroxide were first eluted with 10 ml of chloroform methanol 20:1 v/v. The saturated phospholipids were then eluted with 5 ml of chloroform:methanol:7M ammonium hydroxide 70:30:2 v/v. This eluate was added to 10 ml of Aquasol and counted on a Beckman LS-330 liquid scintillation counter. Lipogenesis experiments were conducted on isolated type II cells and iron containing macrophages which were retained by the magnetic field in the chamber.

**Cell Identification**

Cells were microscopically identified by three methods: fluorescent microscopy with Acridine Orange, modified Papanicolaou staining and electron microscopy. For fluorescent microscopy, unfixed cells were suspended in buffer containing Acridine Orange (1 μg/ml) and viewed through a Nikon fluorescence microscope with excitation at 466 mm and emission at >530. Type II cells were identified by their intensely fluorescing lamellar bodies while other cells fluoresce diffusely green.

Type II cells were identified by modified Papanicolaou staining on the basis of their characteristically dark staining inclusion bodies as described by Kikkawa and Yoneda (64). Air dried
aliquots of cells were stained with a Papanicolaou stain modified by avoiding any fixation with organic solvents before the staining procedure and by omitting an alcohol step in the decolorization. (See Appendix A.)

Cells processed for electron microscopy were fixed in Karnovsky's fixative, post fixed with 2% osmium tetraoxide. After alcohol dehydration, cell pellets were embedded in Spur's plastic (Electron Microscopy Industries, Palo Alto, California) sectioned and stained with uranyl acetate then examined under a Hitachi HS-75 transmission electron microscope.

Additional indication of cell identity was provided by using centrifugal elutriation techniques described by Devereux et al. (62) and Greenleaf et al. (67). Purified cell preparations were introduced into a Beckman JE-6 rotor on a 221-C centrifuge (Beckman Instruments, Inc., Palo Alto, California). Rotor speed was maintained at 2000 rpm with a loading flow rate of 7 ml/min. Cells were eluted and 10 ml fractions collected at stepwise flow rates of 15, 18, 22, 33 and 44 ml/min. The fraction collected at each flow rate was centrifuged at 100 x g for 5 min and the pellet resuspended in 2 ml buffer. Cells in each fraction were identified by modified Papanicolaou staining, fluorescent microscopy with acridine orange and by electron microscopy.
RESULTS

Evolution of Procedure

Enzymatic Dispersion of Lung Matrix

To obtain the proper combination of cell yield and viability (as determined by trypan blue), the effects of various proteases and combinations of these proteases were evaluated by cell count and % viability. Table II contains the results of these experiments.

In some cases up to $45 \times 10^6$ cells were released from the matrix but action of the enzyme was so severe that viability was less than 51%. In these cases, a second experiment was conducted in which the incubation time was reduced to 10 minutes. This was done to determine if viability could be improved. Table III shows that the shorter incubation did increase cell viability, but it also resulted in a decreased cell yield. With a 20 minute incubation of cells in 0.75% trypsin the viability of the released cells was 18% resulting in a yield of $16 \times 10^6$ viable cells/lung. With a 10 minute incubation the viability increases to 88% but yield of viable cells was approximately $12 \times 10^6$ cells/lung.

The results from these preliminary series of experiments indicated that the combination of collagenase and elastase at 0.05% and 1.1 mg%, respectively, represented the best combination of enzymes for dispersing the lung matrix. It then became necessary to determine the optimal time for enzyme activity. In the experiments represented by
Table II. Effectiveness of Various Proteases on Dispersion of Lung Parenchyma from Matrix. Male 200 g Sprague Dawley rats were anesthetized with pentobarbital (50 mg/kg i.p.). The lungs were perfused with Kreb's Ringer Bicarbonate Buffer to remove blood elements. Various combinations of enzyme preparations were instilled into the trachea, then the lungs removed en bloc and minced with sharp scissors to an average block size of 1 mm and incubated in additional enzyme containing buffer for 20 min. When trypsin was used, a soybean trypsin inhibitor (1 mg/ml) and DNAase (30 g/ml) were added to the preparation after 20 min incubations to stop the proteolytic action. To stop other enzyme activities, cells were simply washed. 

<table>
<thead>
<tr>
<th>Enzyme and Concentration</th>
<th>No. of Cells/Lung x 10^6 ±SD</th>
<th>% Viability</th>
<th>No. of Viable Cells/Lung x 10^6 ±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Trypsin</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1%</td>
<td>18 ± 1</td>
<td>78 ± 21</td>
<td>14.04 ± 0.21</td>
</tr>
<tr>
<td>0.25%</td>
<td>28 ± 3</td>
<td>56 ± 18</td>
<td>15.68 ± 0.54</td>
</tr>
<tr>
<td>0.5%</td>
<td>31 ± 6</td>
<td>40 ± 21</td>
<td>12.4 ± 1.26</td>
</tr>
<tr>
<td>0.75%</td>
<td>93 ± 8</td>
<td>18 ± 15</td>
<td>16.84 ± 1.2</td>
</tr>
<tr>
<td>1.0%</td>
<td>107 ± 14</td>
<td>15 ± 11</td>
<td>16.15 ± 1.54</td>
</tr>
<tr>
<td><strong>Thermolysin</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 g/ml</td>
<td>11 ± 4</td>
<td>70 ± 20</td>
<td>7.70 ± 0.8</td>
</tr>
<tr>
<td>20 g/ml</td>
<td>28 ± 3</td>
<td>38 ± 25</td>
<td>10.64 ± 0.75</td>
</tr>
<tr>
<td>30 g/ml</td>
<td>36 ± 11</td>
<td>25 ± 15</td>
<td>9.0 ± 1.65</td>
</tr>
<tr>
<td><strong>Collagenase</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.025%</td>
<td>9 ± 6</td>
<td>95 ± 6</td>
<td>8.55 ± 0.36</td>
</tr>
<tr>
<td>0.05%</td>
<td>18 ± 8</td>
<td>90 ± 4</td>
<td>16.20 ± 0.32</td>
</tr>
<tr>
<td>0.10%</td>
<td>20 ± 10</td>
<td>73 ± 6</td>
<td>14.60 ± 0.60</td>
</tr>
</tbody>
</table>
Table II. Effectiveness of Various Proteases—Continued.

<table>
<thead>
<tr>
<th>Enzyme and Concentration</th>
<th>No. of Cells/Lung x 10^6 ±SD</th>
<th>% Viability</th>
<th>No. of Viable Cells/Lung x 10^6 ±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pronase</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.05%</td>
<td>4 ± 0.8</td>
<td>93 ± 6</td>
<td>3.72 ± 0.048</td>
</tr>
<tr>
<td>0.10%</td>
<td>9 ± 2.0</td>
<td>92 ± 6</td>
<td>8.28 ± 0.12</td>
</tr>
<tr>
<td>0.25%</td>
<td>24 ± 12</td>
<td>73 ± 15</td>
<td>18.25 ± 1.8</td>
</tr>
<tr>
<td>0.50%</td>
<td>30 ± 14</td>
<td>60 ± 11</td>
<td>18.00 ± 1.54</td>
</tr>
<tr>
<td><strong>Enzyme &amp; Concentration</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.55 mg%</td>
<td>0.56 ± 0.3</td>
<td>90 ± 6</td>
<td>0.504 ± 0.018</td>
</tr>
<tr>
<td>1.1 mg%</td>
<td>3 ± 0.6</td>
<td>90 ± 4</td>
<td>2.70 ± 0.024</td>
</tr>
<tr>
<td>2.2 mg%</td>
<td>6 ± 1.1</td>
<td>88 ± 6</td>
<td>5.28 ± 0.066</td>
</tr>
<tr>
<td><strong>Trypsin &amp; Collagenase</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1% 0.05%</td>
<td>15 ± 4</td>
<td>84 ± 7</td>
<td>12.6 ± 0.28</td>
</tr>
<tr>
<td>0.25% 0.07%</td>
<td>21 ± 14</td>
<td>68 ± 8</td>
<td>14.28 ± 1.12</td>
</tr>
<tr>
<td><strong>Trypsin &amp; Pronase</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1% 0.10%</td>
<td>9 ± 6</td>
<td>88 ± 5</td>
<td>7.92 ± 0.3</td>
</tr>
<tr>
<td>0.25% 0.25%</td>
<td>12 ± 3</td>
<td>82 ± 10</td>
<td>9.84 ± 0.3</td>
</tr>
<tr>
<td><strong>Trypsin &amp; Thermolysin</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1% 10 µg/ml</td>
<td>20 ± 15</td>
<td>65 ± 11</td>
<td>13.0 ± 1.65</td>
</tr>
<tr>
<td>0.25% 10 µg/ml</td>
<td>25 ± 14</td>
<td>70 ± 9</td>
<td>17.5 ± 1.26</td>
</tr>
</tbody>
</table>
Table II. Effectiveness of Various Proteases—Continued.

<table>
<thead>
<tr>
<th>Enzyme and Concentration</th>
<th>No. of Cells/Lung x 10⁶ ±SD</th>
<th>% Viability</th>
<th>No. of Viable Cells/Lung x 10⁶ ±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thermolysin &amp; Collagenase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 g/ml 0.05%</td>
<td>8 ± 3</td>
<td>88 ± 6</td>
<td>6.64 ± 8.18</td>
</tr>
<tr>
<td>10 g/ml 0.10%</td>
<td>14 ± 6</td>
<td>80 ± 12</td>
<td>11.2 ± 0.72</td>
</tr>
<tr>
<td>Thermolysin &amp; Pronase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 g/ml 0.1%</td>
<td>16 ± 7</td>
<td>75 ± 15</td>
<td>12.0 ± 1.05</td>
</tr>
<tr>
<td>10 g/ml 0.25%</td>
<td>18 ± 13</td>
<td>70 ± 13</td>
<td>12.6 ± 1.69</td>
</tr>
<tr>
<td>Collagenase &amp; Elastase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.05% 1.1 mg%</td>
<td>27 ± 8</td>
<td>90 ± 10</td>
<td>24.3 ± 0.8</td>
</tr>
<tr>
<td>0.10% 2.2 mg%</td>
<td>28 ± 4</td>
<td>88 ± 10</td>
<td>23.9 ± 0.4</td>
</tr>
<tr>
<td>Elastase &amp; Thermolysin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.1 mg% 10 g/ml</td>
<td>20 ± 6</td>
<td>84 ± 9</td>
<td>16.8 ± 0.54</td>
</tr>
<tr>
<td>2.2 mg% 10 g/ml</td>
<td>28 ± 14</td>
<td>80 ± 11</td>
<td>22.4 ± 1.54</td>
</tr>
<tr>
<td>Elastase &amp; Pronase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.1 mg% 0.1%</td>
<td>14 ± 6</td>
<td>93 ± 6</td>
<td>13.0 ± 0.36</td>
</tr>
<tr>
<td>2.2 mg% 0.25%</td>
<td>19 ± 8</td>
<td>89 ± 4</td>
<td>16.9 ± 0.32</td>
</tr>
<tr>
<td>Trypsin, Collagenase, Pronase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1% 0.05% 0.1%</td>
<td>29 ± 14</td>
<td>84 ± 6</td>
<td>24.3 ± 0.84</td>
</tr>
<tr>
<td>0.25% 0.10% 0.25%</td>
<td>42 ± 20</td>
<td>14 ± 11</td>
<td>5.88 ± 3.8</td>
</tr>
</tbody>
</table>
Table II. Effectiveness of Various Proteases—Continued.

<table>
<thead>
<tr>
<th>Enzyme and Concentration</th>
<th>No. of Cells/Lung x 10^6 ±SD</th>
<th>% Viability</th>
<th>No. of Viable Cells/Lung x 10^6 ±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagenase, Pronase, Thermolysin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.05% 0.1% 10 g/ml</td>
<td>26 ± 21</td>
<td>80 ± 4</td>
<td>20.8 ± 0.84</td>
</tr>
<tr>
<td>0.10% 0.25% 10 g/ml</td>
<td>38 ± 19</td>
<td>65 ± 16</td>
<td>24.3 ± 3.04</td>
</tr>
<tr>
<td>Collagenase, Trypsin, Pronase, Thermolysine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.05% 0.1% 0.1% 10 g/ml</td>
<td>30 ± 15</td>
<td>68 ± 2</td>
<td>20.4 ± 0.3</td>
</tr>
<tr>
<td>0.10% 0.25% 0.25% 10 g/ml</td>
<td>45 ± 23</td>
<td>51 ± 20</td>
<td>22.9 ± 4.6</td>
</tr>
</tbody>
</table>
Table III. Effect of 10 Minute Incubation on Cell Viability. -- Cells were obtained under similar conditions as described for Table II. Incubation time, however, was changed from 20 min to 10 min. n=4, each combination.

<table>
<thead>
<tr>
<th>Enzyme and Concentration</th>
<th>No. of Cells/ Lung x 10^6 ±SD</th>
<th>% Viability</th>
<th>No. of Viable Lung x 10^6 ±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypsin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.75%</td>
<td>13.97 ± 7</td>
<td>88 ± 6</td>
<td>12.3 ± 0.42</td>
</tr>
<tr>
<td>1.0%</td>
<td>13.3 ± 3</td>
<td>75 ± 4</td>
<td>10.0 ± 12</td>
</tr>
<tr>
<td>Thermolysin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 g/ml</td>
<td>12.57 ± 6</td>
<td>63 ± 5</td>
<td>8.0 ± 0.3</td>
</tr>
<tr>
<td>30 g/ml</td>
<td>16.1 ± 9</td>
<td>54 ± 10</td>
<td>9.0 ± 0.9</td>
</tr>
<tr>
<td>Pronase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5%</td>
<td>27.3 ± 11</td>
<td>74 ± 6</td>
<td>20.0 ± 0.66</td>
</tr>
<tr>
<td>Trypsin, Collagenase, Pronase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.25% 0.1% 0.25%</td>
<td>3.9 ± 7</td>
<td>53 ± 8</td>
<td>2.1 ± 0.56</td>
</tr>
<tr>
<td>Collagenase, Trypsin, Pronase, Thermolysin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1% 0.25% 0.25% 10 g/ml</td>
<td>27.17 ± 13</td>
<td>74 ± 14</td>
<td>20.11 ± 1.82</td>
</tr>
</tbody>
</table>
Tables II and III, it was seen that a 50% reduction in time did not reduce cell yield by 50%, but with some combinations of enzymes (Pronase) may have enhanced it. With this in mind, it became important to determine the time required to obtain maximum cell yields. Figure 4 shows the results of these experiments. By 20 minutes of incubation the yield of viable cells had reached a maximum.

At this point, the method of introduction of enzymes into the lung became important. Minced lung preparations provided good contact between cells and enzymes but with these preparations, low percentages of type II cells were released. It was reasoned that with the proper introduction of enzymes to the lungs, a partial enrichment of type II cells could be achieved simply on the basis of selective, discrete dispersion of cells. Experiments were then conducted to determine the best method of introducing enzymes to the lung. Various methods of introducing enzymes to the lung system were compared to each other based on yield of viable cells recovered. Results of these experiments are shown in Table IV.

Once a method was established for removing large numbers of cells from the lungs with enhanced removal of type II cells, it became necessary to develop ways to remove the major cell population contaminating the type II cells. Table V shows results of experiments to determine composition of cells released from rabbit lung matrix by the enzymes. Cells were identified by modified Papanicolaou staining techniques. Type II cells were easily discerned from other cell types but with this stain it was difficult to distinguish between cell types
Figure 4. Effect of Time on Yield of Viable Pneumonocytes.

Lungs from 200 g male Sprague Dawley rats were perfused with Kreb's Ringer Bicarbonate Buffer to remove blood elements. Lungs were then removed en bloc and lavaged 3 times with saline followed by instillation of 0.05% collagenase and 1.1 mg % elastase through the trachea. Parenchyma was then trimmed away from the visible hilar structures and minced with sharp scissors to an average block size of 1 mm. Tissue blocks were then incubated in buffer containing 0.05% collagenease and 1.1 mg % elastase. At each time point, the minced tissue was removed from incubation flasks and filtered through a 40 μm nylon mesh sieve. A sample of the material which passed through the filter was retained for determining yield of viable cells at that time sampling while the rest of the effluent was returned to the incubation flasks along with the minced lung blocks to further incubate until the next time point. Viability was determined with trypan blue exclusion (0.10% ) and counted on a standard hemocytometer.
Table IV. Effects of Various Routes of Administration of Enzymes on Yield of Viable Type II Cells.

Lungs from 200 g male Sprague Dawley rats were perfused with Kreb's Ringer Bicarbonate buffer to remove blood elements. In preparation #1 collagenase 0.05% and elastase 1.1 mg% were perfused through the cannulated pulmonary artery in a recirculatory manner for 10 min. The lungs were then minced and shaken for 10 minutes in a gyrotory water bath at a constant 37°C to remove freed cells. In preparation #2 collagenase and elastase were introduced to the lung via the cannulated pulmonary artery and incubated for 10 min at 37°C. Lung was then minced and shaken for 10 min to remove free cells. In preparation #3 enzymes were introduced to the lung through both perfusion and intra-tracheally. After 20 min incubations the lung was minced and free cells removed. In preparation #4, the enzymes were introduced to the lung through perfusion, then after 20 min of incubations cells were harvested with a syringe attached to the cannulated trachea. In preparation #5 enzymes were introduced to the lungs via cannulated trachea. After 20 minute incubations freed cells were harvested through the trachea. In preparation #6 both perfusion and tracheal instillation of enzymes were used. In preparation #7 lungs were perfused to clear blood elements, then minced and incubated with collagenase and elastase for 20 min. n=2 for each experiment.

<table>
<thead>
<tr>
<th>Minced Lung Preparations #1</th>
<th>Total No. of Cells/Lung x 10^6</th>
<th>% Viability</th>
<th>% Type II</th>
<th>No. of Viable Type II/Lung x 10^6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Perfused with Enzymes</td>
<td>25 ± 6</td>
<td>88 ± 4</td>
<td>28 ± 18</td>
<td>6.16 ± 0.03</td>
</tr>
<tr>
<td>Tracheal Instillation of Enzymes #2</td>
<td>14 ± 5</td>
<td>90 ± 7</td>
<td>44 ± 12</td>
<td>5.54 ± 0.04</td>
</tr>
<tr>
<td>Combination</td>
<td>27 ± 8</td>
<td>90 ± 10</td>
<td>38 ± 13</td>
<td>9.23 ± 0.10</td>
</tr>
<tr>
<td>Tracheal Harvested Cells</td>
<td>2 ± 0.9</td>
<td>90 ± 3</td>
<td>52 ± 8</td>
<td>0.936 ± 0.002</td>
</tr>
<tr>
<td>Perfused/Enzymes #4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table IV. Effects of Various Routes of Administration of Enzymes--Continued.

<table>
<thead>
<tr>
<th>Method</th>
<th>Total No. of Cells/Lung x 10^6</th>
<th>% Viability</th>
<th>% Type II</th>
<th>No. of Viable Type II/Lung x 10^6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tracheal Instillation of Enzymes #5</td>
<td>4.4 ± 1.0</td>
<td>90 ± 4</td>
<td>55 ± 7</td>
<td>2.17 ± 0.002</td>
</tr>
<tr>
<td>Combination #6</td>
<td>6.3 ± 1.5</td>
<td>90 ± 3</td>
<td>49 ± 10</td>
<td>2.77 ± 0.004</td>
</tr>
<tr>
<td>Minced Lung Incubation with Enzymes #7</td>
<td>18 ± 8</td>
<td>90 ± 10</td>
<td>16 ± 15</td>
<td>2.59 ± 0.12</td>
</tr>
</tbody>
</table>
Table V. Major Cell Types Released from Lung Matrix. — Lungs from white New Zealand male rabbits (2-3 Kg) were perfused with Kreb's Ringer bicarbonate buffer and lavaged 3 x with saline to remove free macrophages and surfactant material. Buffer containing 0.05% collagenase and 1.1 mg% elastase was then perfused through the pulmonary artery in a recirculatory manner. In addition, collagenase and elastase were instilled into the lung via the cannulated trachea. The lung was incubated for 10 minutes, then minced and shaken to remove free cells. Monodispersed cells were then sized on a 15 μm nylon mesh filter. A small sample of cells which passed through the filter were prepared for EM and modified Papanicolaou for identification. n=2.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>% of Total</th>
<th>No. of Cells x 10^6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type II Cells</td>
<td>40 ± 14</td>
<td>72 ± 0.9</td>
</tr>
<tr>
<td>Macrophages</td>
<td>33 ± 11</td>
<td>59.4 ± 1.0</td>
</tr>
<tr>
<td>Type I Cells</td>
<td>1 ± 0.02</td>
<td>1.8 ± 0.001</td>
</tr>
<tr>
<td>Endothelial Cells</td>
<td>1 ± 0.0</td>
<td>1.8 ± 0.02</td>
</tr>
<tr>
<td>Ciliated Epithelial Cells</td>
<td>13 ± 0.06</td>
<td>23.4 ± 0.11</td>
</tr>
<tr>
<td>Clara Cells</td>
<td>2 ± 0.9</td>
<td>3.6 ± 1.0</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>1.2 ± 1.0</td>
<td>2.3 ± 3.0</td>
</tr>
<tr>
<td>Eosinophils &amp; Neutrophils</td>
<td>1 ± 1.0</td>
<td>1.0 ± 0.8</td>
</tr>
<tr>
<td>Others — Unidentified</td>
<td>7.8 ± 0.9</td>
<td>14.1 ± 2.3</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>179.4</td>
</tr>
</tbody>
</table>
other than on the basis of size or, as with ciliated epithelial cells, surface morphology. Further cell identification was achieved with electron microscopy which allowed us to examine organelle content and surface morphology of cells. The results in Table V were derived from analysis of the modified Papanicolau stained sections and electron microscopic evaluation. With each method 100 cells were counted and identified. The results from each evaluation were combined for the table and expressed as percent of total cells counted.

Since many cells identified in the preparation were characteristically larger than type II cells it was decided that enhanced purity would be realized by simple sieving the cell preparation through a 15 μm nylon mesh filter. From the data in Table VI it can be seen that many of the contaminating cells remained on the filter while the smaller cells, including type II, passed through and were collected in the effluent.

Since removal of macrophages would result in a relatively homogeneous population of type II cells, it was necessary to develop a simple means of removing macrophages. Magnetic removal of Fe$_3$O$_4$ laden macrophages appeared to be the most effective means available. To determine whether PAMs would take up colloidal Fe$_3$O$_4$, cultured macrophages were incubated in the presence of Fe$_3$O$_4$, then prepared for transmission electron microscopy and examined for evidence of Fe. Figure 5 shows a transmission electron micrograph containing Fe$_3$O$_4$. Deposits of Fe are apparent in discrete areas throughout the cell.
Table VI. Effect on Cell Population Profile of sizing through a 15 μm Nylon Mesh Filter. — Lungs from white New Zealand male rabbits (2-3 Kg) were perfused with Kreb's Ringer bicarbonate buffer and lavaged 3X with saline to remove free macrophages and surfactant material. Buffer containing 0.05% collagenase and 1.1 mg% elastase was then perfused through the pulmonary artery in a recirculatory manner. In addition, collagenase and elastase were instilled into the lung via the cannulated trachea. The lung was incubated for 10 min then minced and shaken to remove free cells. Mono-dispersed cells were then sized on a 15 μm nylon mesh filter. A small sample of cells which passed through the filter were prepared for EM and modified Papanicolaou for identification. n=2.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>% of Total</th>
<th>No. of Cells x 10^6 Passing through Filter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type II Cells</td>
<td>58</td>
<td>60 ± 14</td>
</tr>
<tr>
<td>Macrophages</td>
<td>30.5</td>
<td>30 ± 8</td>
</tr>
<tr>
<td>Type I Cells</td>
<td>&lt;1.0</td>
<td>&lt;1 ± 0.001</td>
</tr>
<tr>
<td>Endothelial Cells</td>
<td>&lt;1.0</td>
<td>&lt;1 ± 0.001</td>
</tr>
<tr>
<td>Ciliated Epithelial</td>
<td>&lt;1.0</td>
<td>&lt;1 ± 0.018</td>
</tr>
<tr>
<td>Clara Cells</td>
<td>&lt;1.0</td>
<td>&lt;1 ± 0.021</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>2.3</td>
<td>2.3 ± 0.9</td>
</tr>
<tr>
<td>Eosinophils &amp; Neutrophils</td>
<td>&lt;1.0</td>
<td>&lt;1.0 ± 0.133</td>
</tr>
<tr>
<td>Others — Unidentified</td>
<td>&lt;1.0</td>
<td>&lt;1.0 ± 1.0</td>
</tr>
<tr>
<td>Total</td>
<td>96.8</td>
<td>98.3</td>
</tr>
</tbody>
</table>
Pulmonary alveolar macrophages were lavaged from the lungs of a 2.2 Kg white New Zealand male rabbit and cultured in a 33 mM petri dish with medium 199 for 2 hrs at 37°C under 95% O2 5% CO2. Non-adhering cells were washed away with fresh medium. 300 µl of 0.99 mg/ml solution of Fe3O4 were added to each dish and incubated for 10 minutes. Medium including excess Fe3O4 was washed off at this time and cells lightly trypsinized to remove from the dish. Cells were then washed and prepared for EM. The results from experiments to determine optimal time for uptake of Fe3O4 are shown in Fig. 6. 7000X.
Since macrophages could accumulate Fe₃O₄ particles, experiments were then conducted to determine the optimal time for uptake. The data from these experiments are presented in Figure 6. Accumulation of Fe₃O₄ was linear for approximately 10 minutes and reached a maximum accumulation at 20 minutes. From this work it was decided that optimal incorporation of Fe₃O₄ into the macrophages could be achieved in 30 minutes.

To achieve separation of Fe₃O₄ laden macrophages from other cells in the preparation a strong magnetic field was applied to the cell suspension. In the first experiments to show this, Fe₃O₄ containing macrophages were added dropwise to a concave microscope slide which contained a magnetic probe. Figures 7, 8 and 9 show the results of these experiments which demonstrate the attraction of Fe₃O₄ containing macrophages to a magnetic field.

The second experiment conducted to demonstrate magnetic attraction of cells was to add Fe₃O₄ containing cells to a specially prepared plastic cuvette (Figure 10). When placed in a light spectrophotometer, a decrease in absorbance was demonstrated as cells were attracted to the magnetic strips in the cuvette.

Once it was established that macrophages could effectively be removed, development of this method for isolating type II cells was essentially complete. A chamber was constructed for continuous removal of magnetic macrophages and the procedures which evolved from the preliminary experiments for removing contaminating cell lines were placed into a series of procedures as described in Materials and Methods.
Figure 6. Effect of Time on Uptake of Magnetite by Pulmonary Macrophages.

Pulmonary alveolar macrophages were lavaged from a 2-3 Kg white New Zealand male rabbit and cultured in 35 mM petri dish with 5 mls of medium 199 for 2 hrs at 37°C under 95% O₂ 5% CO₂. Non-adhering cells were washed away with fresh medium. 300 μl of 0.9 mg/ml Fe₃O₄ solution were added to each dish. Cell samples were taken at each time point by washing away excess Fe₃O₄ then gently trypsinizing the cells off the dishes and preparing cells for EM. n = 2.
Figure 7. Magnetic Probe before Addition of Macrophages.

Macrophages were harvested from white New Zealand male rabbits (2-3 Kg) by lavage. Cells were then incubated in medium 199 for 2 hrs, then washed with fresh medium to remove non-adhering cells. 300 μl of 0.99 mg/ml colloidal Fe₃O₄ were added and the preparation incubated for 10 minutes. Cells were lightly trypsinized to remove cells, then washed 1 x in fresh medium. A concave microscope slide containing a magnetic probe was placed under a standard light microscope. The tip of the probe can be seen in the lower right corner. Figure 7 shows the preparation before addition of cells. 250X.
Figure 8. Magnetic Probe Immediately after Addition of Macrophages.

This figure is a picture taken within 15 seconds of addition of cells. 250X.
Figure 9. Magnetic Probe 1 Minute after Addition of Macrophages.

This figure shows the preparation 1 minute after addition of cells. Note that all cells are attracted to the probe. 250X.
Figure 10. Removal of Macrophages Containing Fe₃O₄ as Measured Decreased Absorbance at 600 nm.

Macrophages laden with magnetite were added to a plastic cuvette shown above. On double beam mode the Beckman Lighe UV spectrophotometer ACTA V traced absorbance at 600 nm vs time with cells in both reference and sample cuvette. As can be seen from the 1st tracing, settling in both cuvettes was similar as displayed by the horizontal trace. When a magnet was attached to the sample cuvette absorbance decreased rapidly, shown in 2nd trace.
These standardized procedures were utilized in the remainder of experiments presented in this Results section.

Characterization of Cells

Cell Yield and Purity

Summary of type II cell yield per rabbit is presented in Table VII. Large variations in yield existed from one experiment to another which is reflected in the large standard deviations presented. It was found that the best cell recovery occurred in experiments when initial pulmonary perfusion rapidly cleared blood elements from the lung. When clearance of blood elements was slow the recovery of cells decreased. The results of yield reported in Table VII were compiled from 20 consecutive experiments and included data points from animals that did not perfuse rapidly. Variability in yield was reduced when recovery values from well perfused lungs exclusively were compiled.

Figure 11 compares relative purity and yield of cells of methods 1 and 2. Method 1 produced higher purity but low cell numbers. Method 2 yielded high cell numbers but of lesser purity than method 1. The purity of cells obtained from method 1 was encouraging for experiments requiring very pure preparations but for many experiments the low yield of cells made method 1 undesirable. Method 2, although not as enriched in type II cells, produced substantially more cells making it practical for routine procedures in primary culture experiments.
Table VII. Summary of Characteristics of Cells Isolated by Recirculatory Perfusion, Sizing and Magnetic Macrophage Removal.

<p>|                               | Method #1 | Method #2 |</p>
<table>
<thead>
<tr>
<th></th>
<th>Cell Digest</th>
<th>Purified</th>
<th>Cell Digest</th>
<th>Purified</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total Cells</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\times 10^6$</td>
<td>$20 \pm 7$</td>
<td>$8 \pm 2.1$</td>
<td>$180 \pm 40$</td>
<td>$36 \pm 8$</td>
</tr>
<tr>
<td><strong># of Type II Cells</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\times 10^6$</td>
<td>$11 \pm 3$</td>
<td>$7.6 \pm 1.4$</td>
<td>$37 \pm 9$</td>
<td>$32 \pm 7$</td>
</tr>
<tr>
<td><strong>% Type II Cells</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>$55$</td>
<td>$95$</td>
<td>$21$</td>
<td>$88$</td>
</tr>
<tr>
<td><strong>Viability</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>$96$</td>
<td>$93$</td>
<td>$94$</td>
<td>$90$</td>
</tr>
<tr>
<td><strong>O₂ Consumption</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nmoles $O₂/10^6$ Cells/Hr</td>
<td>$84 \pm 4$</td>
<td></td>
<td>$88 \pm 6$</td>
<td></td>
</tr>
<tr>
<td><strong>$^{14}$C Acetate Incorp.</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nmoles $/10^6$ Cells/Hr</td>
<td>$0.44 \pm 0.1$</td>
<td>$0.18 \pm 0.06$</td>
<td>$0.43 \pm 0.1$</td>
<td>$0.18 \pm 0.06$</td>
</tr>
<tr>
<td><strong>$^{14}$C Choline Incorp.</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nmoles $/10^6$ Cells/hr</td>
<td>$0.115 \pm 0.01$</td>
<td>$0.030 \pm 0.005$</td>
<td>$0.115 \pm 0.01$</td>
<td>$0.030 \pm 0.005$</td>
</tr>
</tbody>
</table>

1) n=20
2) n=6
Significance: Differences exist between the incorporation rate of acetate or choline in type II cells and incorporation rates in isolated macrophages as evaluated by unpaired Student's t. p <0.01.
Method 1 consisted of freeing cells from lung matrix using collagenase and elastase 0.05% and 1.1 mg %, respectively, then harvesting cells through the trachea. These results are labeled "TRACHEAL LAVAGE AFTER COLLAGNEASE" on the graph. Method 2 freed cells by a combination of collagenase and elastase with a physical disruption of the lung by mincing the lung with sharp scissors. On the bar graph above these data are found on "MINCED LUNG COLLAGENASE DIGEST" columns.
In both preparations, high initial levels of type II cells (55-68%) indicate the enzyme preferentially released these pneumocytes from the lung matrix. This suggests that the application of enzymes at the alveolar region is in itself an enrichment procedure since application of enzymes to the minced lung releases type II cells in initial concentrations of only 10-30% (Table IV).

Table VIII describes the nature of cellular contaminants. Qualitatively, cells contaminating the preparation were equivalent; however, contamination from ciliated cells was significantly different between method 1 and 2, and was consistent in each series of experiments.

Cell Viability

Evaluation of cell viability showed greater than 90% of the isolated cells from both procedures excluded trypan blue. Since exclusion of trypan blue is not an absolute indication of viability other means were used to evaluate cell viability.

$O_2$ consumption of the isolated type II cells was compared to $O_2$ consumption of macrophages recovered from the separation chamber magnets (Figure 12). $O_2$ consumption of the type II cells was 88.32 nmoles $O_2/10^6$ cells/hr while macrophages consumed $O_2$ at the rate of 206.00 nmoles $O_2/10^6$ cells/hr. The addition of 10 mM Na succinate did not significantly alter the rate of $O_2$ consumption by the cells when results were evaluated by paired Student’s t test. This indicates cell membranes were intact and impermeable to Na succinate further suggesting good viability.
Table VIII. Relative Nature of Contaminants in Purified Type II Preparations. — These values represent the relative distribution of contaminants in type II preparations ± standard deviation. Cell types were identified by transmission electron microscopy. With each preparation, 100 cells were counted and percentages based on data from these EM examinations were compiled for this table. Mann-Whitney U Test comparison between Method 1 and 2 demonstrated significant differences in populations of non type II cells only in ciliated epithelial cell contamination. n=20.

<table>
<thead>
<tr>
<th></th>
<th>Percentage of Non Type II Cells in Preparations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Macrophages</td>
</tr>
<tr>
<td>Purified Method 1</td>
<td>9 ± 6</td>
</tr>
<tr>
<td>Purified Method 2</td>
<td>13 ± 8</td>
</tr>
</tbody>
</table>

* Signif. <p 0.01.
Figure 12. Oxygen Consumption of Isolated Type II Cells.

Type II cells were obtained by methods described in this report. 10 x 10⁶ cells were placed in a 1.8 ml Clark type O₂ electrode with HEPES buffer and 300 1 fetal calf serum. After a 5 minute equilibrium period O₂ consumption was measured during 10 minute periods. During one of these measurements the total chamber concentration of Na Succinate was brought to 10 mM and O₂ consumption measured. These values for O₂ consumption were compared to Fe₃O₄ containing macrophages which were taken from the separation chamber after type II cells enrichment. Values were expressed as n moles O₂ consumed/10⁶ cells/hr.
Viability of cells was also evaluated by their ability to incorporate radiolabeled choline and acetate into the disaturated lecithin components of chloroform-methanol extractable lipids. Figure 13 shows results from these experiments. Incorporation of $^{14}\text{C}$ Na acetate or $^{14}\text{C}$ choline chloride was linear for over two hours. Comparison of acetate and choline incorporation in macrophages was conducted as a control for the disaturated lecithin assay. When expressed on per cell basis, the incorporation of acetate into disaturated lecithin was 40% greater with type II cells than with macrophages while choline incorporation was 26% greater in type II cells compared to macrophages (non-paired Student's $t$ $p=0.05$, $n=6$).

Isolated cells appeared normal upon examination with transmission electron microscopy. Most mitochondria and cell membranes did not appear swollen or damaged, although with method 2 a number of cells were observed which presented slightly swollen appearances which was considered reversible. All cells resembled those found in the intact lung (Figure 14).

Cell Identity

Cells were identified as described in Materials and Methods and as shown in Figures 15a,b, 16a,b, and 17. In addition cells were identified on the basis of elutriation head separation also described in Materials and Methods.

When cells were separated on the elutriator, 86% eluted at 22 ml/min flow rate. Ninety-eight percent of these were type II cells. Lesser amounts of type II cells were found at other flow rates as seen
Type II cells were isolated by methods described in this report. 5 x 10^6 cells were incubated with either 5 μCi ¹⁴C Na Acetate (8 μCi/μmole) or 5 μCi ¹⁴C Choline Chloride (21 μCi/μmole) for 3 hours. The reaction was stopped by adding 20 volumes of Folch reagent and the lipids separated according to methods described. Rates of incorporation were determined and expressed as nM/10^6 cells/hr. Rates were compared with non-paired Student T. n = 6.
Figure 14. Transmission Electron Micrograph of Isolated Type II Cells in A Field of 3 Cells.

For electron microscopy pelleted cells were washed in cacodylate buffer then osmicated for one hour at 4°C. Cells were then again washed then stained with uranyl acetate for 15 minutes. Following graded ethanol dehydration, cells were embedded in Spur's, thin sectioned and examined with an Hitachi HS 7S transmission electron microscope. 2300X.
Figure 15. Modified Papanicolaou Stained Cells.

Figure a shows a preparation of type II cells stained with modified Papanicolaou stain. Cells appeared granular with darkly staining inclusion bodies thought to be the surfactant containing lamellar bodies characteristic of type II cells. Fig. b is a mixed preparation of cells, almost exclusively pulmonary alveolar macrophages. They appear larger and stain more uniformly blue. 250X.
Figure 15. Modified Papanicolaou Stained Cells.

a Type II cells

b Mixed Preparation
Figure 16.  Fluorescence Microscopy of Type II Cell Preparations.

Cells were isolated according to procedures described in the materials and methods section. For identification with fluorescence microscopy cells were incubated for 5 min. at room temperature with 1 µg/ml Acridine Orange and viewed through a Nikon fluorescence microscope with excitation energy at 466 nm and emission at 530. Type II cells were identified by their intensely fluorescing appearance as compared to macrophages which displayed more granular diffusely fluorescing properties. A number of macrophages can be observed in this preparation. For comparison a phase contrast micrograph is shown in figure b. Both show the same field of cells. 250X.
Figure 16. Fluorescence Microscopy of Type II Cell Preparations.
Figure 17. Transmission Electron Microscopy of an Isolated Type II Cell.

Cells were prepared for electron microscopy as described in Material and Methods. Characteristic osmophillic lamellar bodies can be seen. Cells contain large amounts of lipid materials giving this cell a vaculated appearance as observed with electron microscopy. 7000X.
in Table IX. The use of elutriation helped confirm the identity of the cells isolated since published elutriation methods (62,67) indicate the majority of type II cells elute at flow rates between 18 and 22 ml/min. Other elution flow rates produce fractions of contaminating cells according to size. Less than 10% of the cells loaded into the system eluted at flow rates other than 22 ml/min. This was interpreted as evidence of homogeneity of cells introduced to this system.
Table IX. Elutriator Cell Fraction Analysis of Isolated Type II Cells. — 38 x 10^6 cells recovered from the isolation procedure described in this report were further separated on a Beckman JE-6 elutriator rotor and each fraction evaluated by EM, modified Papanicolaou and fluorescence as indicated in Materials and Methods. n=2.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Flow Rate (ml/min)</th>
<th>Number of Cells x 10^6</th>
<th>% Type II</th>
<th>Nature of Major Cell Population in Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7</td>
<td>38</td>
<td>—</td>
<td>Loading</td>
</tr>
<tr>
<td>2</td>
<td>15</td>
<td>1.6 ± 0.2</td>
<td>1</td>
<td>Cell debris, lymphocytes, RBCs, some type II</td>
</tr>
<tr>
<td>3</td>
<td>22</td>
<td>26 ± 5</td>
<td>98</td>
<td>Type II, macrophages</td>
</tr>
<tr>
<td>4</td>
<td>35</td>
<td>1.2 ± 0.8</td>
<td>30</td>
<td>Macrophages and some Clara cells</td>
</tr>
<tr>
<td>5</td>
<td>44</td>
<td>0.4 ± 0.1</td>
<td>8</td>
<td>Clara and some macrophages</td>
</tr>
</tbody>
</table>
DISCUSSION

These results demonstrate that a rapid and effective procedure for enriching type II cell lung cell fractions has been developed. This method includes three basic techniques to remove contaminating cell fractions from mono-dispersed lung preparations without differential gradient centrifugation. Past methods of isolating alveolar type II cells relied on density or size differences between cells to achieve separations. Where large size or density differences exist between cells, these procedures afford good separation. In the lung, however, type II and macrophage cell sizes and densities are too similar for complete separation. To overcome this, other investigators describe preparations of mono-dispersed pneumocytes made in the presence of colloidally suspended dense materials such as BaSO₄ or Fluorocarbon Albumin. This simple maneuver allowed the macrophages to phagocytize heavy materials which altered densities of the phagocytic cells. Even though this manipulation of macrophages improved separation, contamination still occurred.

To avoid this problem, the procedure described here was developed in which cells were prepared in the presence of colloidally suspended magnetic iron oxide. In this way, macrophages containing iron oxide could be removed through the influence of a magnetic field. This allowed removal of macrophages based on the magnetic characteristics of the cells, not on size or density. Electron microscopic
examination of type II enriched fractions indicated little contamination from macrophages which contained visible iron oxide. However, a small percentage of cells (5-10%) could be identified as macrophages without iron oxide. We assumed these cells to be non-viable since they did not internalize magnetic particles. In studies using cells isolated by the method described here, non-viable cells, particularly at such a low concentration, should not interfere with the outcome of most experiments requiring isolated type II cells.

Macrophages, types I and II epithelial cells, lymphocytes, and endothelial cells constitute the major cell types in unpurified monodispersed lung cell fractions prepared with the method described in this report. Magnetic sequestration removes macrophages but lymphocytes endothelial cells and type I epithelial cells need to be removed to significantly enrich the preparation in type II cells. Endothelial cells were removed early in the recirculatory lung perfusion with collagenase and elastase. Since they do not tenaciously adhere to the basement membrane, gentle treatment with the enzymes perfused through the vasculature removed the endothelial cells and carried them out of the lung with the perfusion medium.

Reports of methods which used rat preparations have indicated that perfusion through the pulmonary artery to remove endothelial cells was not successful (82). Apparently the loosened endothelial cells collected in capillary regions and blocked these areas, thereby occluding major portions of the lung. We did not find this a problem in rabbit preparations. When elastase-collagenase concentrations were
maintained at 0.1 mg/100 ml and 50 mg/100 ml, respectively, perfusion proceeded without occlusions. In earlier experiments, excessive enzyme concentrations caused blockage, presumably because large quantities of cells were suddenly released from the endothelium. When the proper enzyme concentrations were used, endothelial cells were released from the basement membrane slowly enough to be cleared from capillaries without aggregation and blockage.

In rat preparations, it is possible that optimal enzyme concentrations would not prevent blockage due to physical limitations in rat lungs not apparent in rabbit lungs. This obstacle could be overcome in rat preparations by cannulating both the pulmonary artery and vein. Perfusion through the pulmonary artery would remove endothelial cells and force them out of the lung through the pulmonary vein cannula. If blockage occurred, then simply perfusing the lung from the pulmonary vein cannula would force the endothelial cells back out of the lung through the pulmonary artery. Further removal of endothelial cells was accomplished during filtration of monodispersed cells through the 15 μm sieve. Thus, in most preparations, endothelial cells could be effectively removed without the use of differential gradient techniques.

A further advantage of perfusing enzymes through the pulmonary artery was the discrete application of enzyme to desired sites of action. Methods in the past have applied dispersing enzymes to minced lung preparations allowing the enzymes to affect all 40 cell types in the lung (62,64,66,67). In this new procedure, dispersing enzymes gain direct access to epithelial cells once the perfusate crosses the
basement membrane and this direct access results in preferential
dissociation of epithelial cells.

Type I cells were removed from the preparation by simply
sieving the suspension through a 15 μm nylon mesh filter. Because type I epithelial cells are very large (8) (40-80 μm), they do not readily pass through the filter while smaller cells including type II can pass.

Throughout the cell separation procedures, it was important to maintain cells in a mono-dispersed suspension. Clumping of cells interfered with not only the effectiveness of filtering and magnetic separation of cells but reduced yields greatly. As a partial solution to this clumping separation procedures were conducted in the cold (4°C) since cell aggregation has been shown to be a function of temperature (83). As a further measure to reduce clumping, serum was added to all buffers. Protein contained in the serum may have bound to surface receptor sites which mediate cell aggregation. The combination of separation in the cold and the addition of protein to the buffers effectively prevented cell clumping and helped improve yield.

Results from this study further demonstrate viability and purity as well as yield per animal were similar to those reported by other groups (62,64,66,67). Viability determinations with trypan blue were used only to grossly screen cell populations. Oxygen uptake and radio-labeled precursor experiments further characterized viability. Results from these experiments show cells isolated with this procedure consume oxygen at a rate less than cells isolated by the density gradient centrifugation and elutriation method of Greenleaf, Mason and Williams (66).
An examination of the nature of the contaminants from these procedures would help explain the differences in $O_2$ uptake. With techniques to isolate type II cells based on density differentiation, the major contaminant has been macrophages which have a very high rate of $O_2$ uptake. This is reflected in the high rate of $O_2$ uptake in type II preparation produced by density differential techniques. With our method for isolating type II cells, the major cellular contaminants were lymphocytes which have a low rate of respiration (84). Therefore, $O_2$ consumption would appear lower in the preparation contaminated with lymphocytes.

Lipid uptake experiment values were also similar to published reports with one interesting difference. Mason's group and others (64,66,67) reported cells required 30 min-1 hr of incubation after isolation before linear uptake of radiolabeled precursors could be demonstrated. This incubation period was postulated necessary to allow cells recovery of normal metabolic function after disruption by the isolation procedure. With the isolation procedure described in this report, linear uptake of radiolabeled precursors could be shown immediately after isolation of cells, eliminating the need for a lengthy recovery period. This may be due, in part, to the rapidity with which the cells were isolated. Other procedures require 3-5 hrs for separation and in that time some reversible metabolic alterations could occur. The method described in this report requires less than two hours and does not require the cells to be subjected to lengthy centrifugations, thereby reducing the possibility of damage occurring.
Further, it is possible that enzymes used for dispersing the pneumocytes may be responsible for recovery time differences between procedures. Finkelstein and Mavis (79) reported alterations in membrane receptors after treatment with trypsin which were distinct from membrane receptor alterations caused by collagenase. Since our method uses collagenase and elastase to remove cells from the matrix, it is conceivable that cellular damage caused by this method has less effect on lipid uptake and synthesis than methods which use trypsin. With this assumption it would follow that less recovery time was required for collagenase treated cells because less damage to lipid metabolism occurred.

The procedure described in this report has the advantage of not using differential centrifugation but because of this, one major disadvantage has become obvious. With differential centrifugation techniques most cell lines released by the dispersing enzymes partition into areas of the gradient away from the area where type II cells and macrophages were recovered. In this new procedure, however, if cells other than magnetic macrophages, type I or type II epithelial were released from the lung matrix there was no provision for removing them from the preparation. For example, despite discrete application of enzymes to epithelial areas, cells which occupy lower terminal bronchioles or respiratory bronchioles may come into contact with enzymes long enough to be removed with epithelial cells. As another example, the major cellular contaminants of our preparations have been lymphocytes. Lymphocytes were found in preparations, despite thorough perfusion of the intact lung. With elutriation techniques, both of
these sources of contamination can be separated from type II cells based on their differences in size. Our method of isolation provides no means of removing these cells. Fortunately, lymphocytes possess very little drug metabolic capabilities, therefore, contamination from this source would cause minimal concern in drug or xenobiotic metabolic studies. However, the lower terminal bronchioles contain Clara cells which have been shown to possess greater xenobiotic metabolic capabilities than any other cell line within the lung (68). Excessive contamination from these cells may alter metabolic experiments requiring purified type II cells. Therefore, in developing this new procedure for isolating type II cells, we hoped that the 15 μ filter would, in addition to removing epithelial type I cells, also remove Clara cells which reportedly had diameters ranging from 25-40 μm (1,68). Unfortunately, even with the filter, Clara cells were found in the preparations. This may be due in part to the range in sizes of Clara cells which would allow smaller Clara cells to pass through the filter along with type II cells.

In future experiments it would be possible to combine the magnetic separation of cells and elutriation methods to produce cell preparations free of Clara cells. In our experiments with the elutriator, cell fractions were obtained which were free from both Clara cells and lymphocytes. The major disadvantage of this approach would be the time element. The elutriator preparation required an additional 1 hr for completion. In this time the advantages from a rapid isolation procedure would be lost, but may be necessary for
definitive studies where contaminating Clara cells would present a problem.

In this study, we conclude that contamination from non-magnetic cells was of minimal importance since electron microscopy showed the majority of contaminants were not Clara cells and would not interfere with the drug metabolic characterization of type II preparations. In addition, the purity of preparations made with this new procedure was similar to purity reported by other methods which were used in measuring metabolic capabilities of isolated type II cells.

The potential advantage of this new procedure is its rapidity. Previously reported methods for isolating type II cells required 4-6 hrs of preparation. In this time metabolic alterations may be taking place which could contribute to cellular responses different from those in the intact animal. Numerous groups have reported biochemical and morphological alterations in type II cells in culture (72, 74, 75, 78). Since it is not known what causes these alterations or even when they initiate, it becomes important to isolate the cells rapidly. With this new procedure, type II cells can be removed from the lung and purified in less than 2 hours. A method which would allow rapid removal and purification of pneumocytes has the advantage of providing cells for study which may possess metabolic functions more like type II cells in the intact animal.
Future Work

As is the case in many projects, bench work halted with questions yet unanswered. In the preliminary phases with enzyme dispersal of parenchyma, rats were used to minimize cost. However, when the supply of healthy rats became unavailable, rabbits were used. A number of experiments were repeated to determine if data from rat studies were valid for rabbit models. These repeated experiments were abbreviated versions of the originals and because of this, all experiments done in rats initially should be conducted on rabbits. Even though there is little reason to believe results will be significantly different, the possibility exists that the optimal enzymes for dispersing rabbit lung parenchyma may be different from those found to work in the rabbit.

The test of any newly developed procedure is in its application. To truly test this method for isolating type II cells, the procedure should be used in experiments designed to evaluate some function of type II cells. In toxicological work the effects of various toxic agents on type II cells could be partially characterized through alterations in surfactant production — qualitatively or quantitatively. Levels of reduced glutathione levels or SOD or any other of a number of peroxidases in type II cells could provide valuable data for evaluation of the toxic effects of agents. Pharmacological evaluations on isolated type II cells could help determine mechanisms of action of hormones or other drugs which have been shown to cause increased or decreased surfactant syntheses in whole animal experiments. Specific uptake of
basic amines have been reported in the lung (85) as well as uptake of sulfates and sulfites (86). With isolated type II cells, it could be determined if these compounds were being taken up into type II cells or other fractions of the lung. Since it has been shown that type II cells possess some drug metabolic capabilities (62), it would be appropriate to study the metabolism of various compounds by isolated cells.

To carry this project further, it may be possible to use techniques developed here to isolate other cell fractions from the lung. Most procedures now available depend upon culturing or density gradient centrifugation -- both of which are very time consuming. There exists room for development of rapid methods for isolating other cell types in the lung; Clara cells, endothelial cells, type III or J cells, ciliated bronchiole cells and many others of the over 40 cell types.
APPENDIX A

METHOD FOR MODIFIED PAPANICOLAOU STAIN
Cyto centrifuge slides, unfixed, air-dried

1. Dip slides in hematoxylin and let sit for 3 minutes
2. Rinse slides in distilled water two or three times until they no longer shed stain
3. Lithium carbonate solution for 2 minutes
4. Rinse slides in distilled water
5. 50% ethanol for 1-2 minutes
6. 80% ethanol for 15 seconds
7. 95% ethanol for 15 seconds
8. Orange G 6 for 1 minute
9. 95% ethanol rinse
10. 95% ethanol rinse
11. 95% ethanol rinse
12. EA 50 for 1 minute
13. 95% ethanol rinse
14. 95% ethanol rinse
15. 95% ethanol rinse
16. Absolute ethanol for 30 seconds
17. Xylene:absolute ethanol 1:1 for 30 seconds
18. Xylene rinse
19. Xylene rinse. Slides can stay in xylene for 30 minutes-1 hour before mounting.
20. Coverslip and mount with Permount
APPENDIX B

FLOW DIAGRAM OF PROCEDURE
Recirculatory Perfusion of Rabbit Lung
With HEPES
With 10% Fetal Calf Serum

\[ \downarrow \]

3 X Tracheal Lavage

\[ \downarrow \]

Instillation of Coloidal Magnetite
Followed by 30 min Incubation

\[ \downarrow \]

Perfusion and Instillation of HEPES Buffer
Containing Elastase and Colagnease
Followed by 10 min Incubation

Method #1
\[ \downarrow \]
Harvest of Cells Through Trachea
\[ \downarrow \]
Filter Through 15-μm Nylon Mesh Sieve
\[ \downarrow \]
Passage Through Separation Chamber
\[ \downarrow \]
Type II Cells 95% Pure

Method #2
\[ \downarrow \]
Mincing of Lungs and 10 min Shaking in 37° C Water Bath
\[ \downarrow \]
Filtering Through 15-μm Nylon Mesh Sieve
\[ \downarrow \]
Passage Through Separation Chamber
\[ \downarrow \]
Type II Cells 88% Pure
REFERENCES


17. Evans, M. J., Cabral, L. J., Stephens, R. J., Freman, G. Transformation of Alveolar Type II Cells to Type I Cells Following Exposure to NO2. Exp. and Molecular Path. 22:142, 1975.


65. Personal Communication. Dr. Theodora R. Devereux, Laboratory of Pharmacology National Institute of Environmental Health Sciences, P. O. Box 12233, Research Triangle Park, NC 27709.


73. Mason, R. J., and Williams, M. C. Phospholipid Composition and Ultrastructure of A-549 Cells and Other Cultured Pulmonary Epithelial Cells of Presumed Type II Cell Origin. *Biochimica et Biophysica Acta.* 617:36, 1980.


