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**STUDIES ON PULMONARY ALVEOLI:
THE EFFECT OF LUNG VOLUME AND OF HYPERVENTILATION**

**Submitted as a thesis for the degree of
DOCTOR OF PHILOSOPHY
to the Medical Faculty of the
UNIVERSITY of GLASGOW**

BY

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LAMMAS, 1969.**

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SUMMARY OF THESIS :

STUDIES ON PULMONARY ALVEOLI:

THE EFFECT OF LUNG VOLUME AND OF HYPERVENTILATION

The surface tension properties of the internal lung surface play a significant role in the mechanics of respiration, by promoting stability of the air spaces. Hyperventilation has been shown by other workers to reduce pulmonary surface activity and cause instability of the alveoli. During the course of this study it was realised that the shape and size of alveoli were of great importance when evaluating alveolar stability, and that much more work was needed to establish the dimensional changes of alveoli occurring during the respiratory cycle, in the normal lung.

In this thesis the methods available for studying pulmonary surface activity are reviewed and the evidence that a specific surfactant is responsible for the peculiar surface tension properties of the lungs, is critically examined. The few existing publications of studies on the shape and size of alveoli are assessed.

PART 1 deals with studies on pulmonary surface activity in the normal lung and following a period of hyperventilation, in the guinea pig. Several methods were used,

1. pressure-volume manoeuvres during air filling.
2. measurement of lung compliance and calculation of the lung stability index.
3. examination of stable lung bubbles and the calculation of

bubble stability ratios.

4. fluorescent microscopy for assessing lung autofluorescence and the fluorescence of stable lung bubbles.

RESULTS:

Hyperventilation caused the following ,

1. the deflation curve of the pressure-volume relationship was less sigmoid, and the pressure required to maximally inflate the lungs was increased.
2. lung compliance was reduced by 30% .
3. the lung stability index was reduced by 17% .
4. the lung bubble stability ratio was reduced by 11% .
5. autofluorescence of frozen unfixed sections of lung tissue was reduced, but the fluorescence of stable lung bubbles was unaltered.

Thus hyperventilation caused an increase in the forces which to be overcome during lung inflation and decreased the stability of the air spaces, resulting in a tendency to premature collapse of the lungs.

PART 11 deals with the measurement of the shape and size of alveoli and alveolar ducts on thin microscopic sections of the lungs of guinea pigs which had been rapidly frozen.

A technique was developed by which living anaesthetized guinea pigs could be artificially ventilated with a positive and negative cycle, and rapidly frozen at the instant of cessation of ventilation at a preselected point on the respiratory cycle. The morphometric

methods used are described in detail and the volume shrinkage due to processing of the lungs was evaluated.

Two sets of experiments were carried out using these techniques. The normal respiratory cycle was interrupted at different degrees of inflation (1) after spontaneous breathing, (2) after a period of hyperventilation.

RESULTS:

The total number of alveoli as well as the total alveolar surface area were found to depend on body weight.

Effect of lung volume.

1. the total volume of alveoli increased during lung inflation.
2. the total volume of alveolar ducts changed little during lung inflation until the lungs were 40% inflated; thereafter it increased steeply.
3. the total alveolar surface area increased steeply during lung inflation from low lung volumes, but levelled off when the lungs were more than 50% inflated.
4. the volume to surface ratio of alveoli was unaltered during lung inflation.

Effect of hyperventilation.

1. the total alveolar volume was reduced but the total alveolar duct volume was unaltered.
2. the total alveolar surface area was reduced. The reduction was most marked at high degrees of lung inflation.

The above effects of hyperventilation were related to the duration of hyperventilation.

Electron microscopic evidence is produced demonstrating that the alveolar surface membrane is smooth whether the lungs are fully inflated or whether they are collapsed. The harmonic mean thickness of the air-blood barrier was decreased by 33% when the lungs were fully inflated from a near collapsed state.

The functional significance of these findings is discussed with particular reference to the role of pulmonary surfactant.

ACKNOWLEDGEMENTS

Dr Sheila Jennett, has provided throughout invaluable practical advice and encouragement. She has shown, by having a remarkable facility of seeing the basics of a problem quickly, and producing a sound method of approach to its solution, that she is indeed an expert scientist. I am deeply indebted to her for the opportunity of working with her.

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Finally, my wife, Janette, has throughout given encouragement, and borne with good humour the difficulties, attendant with ones pre-occupation with ones work.

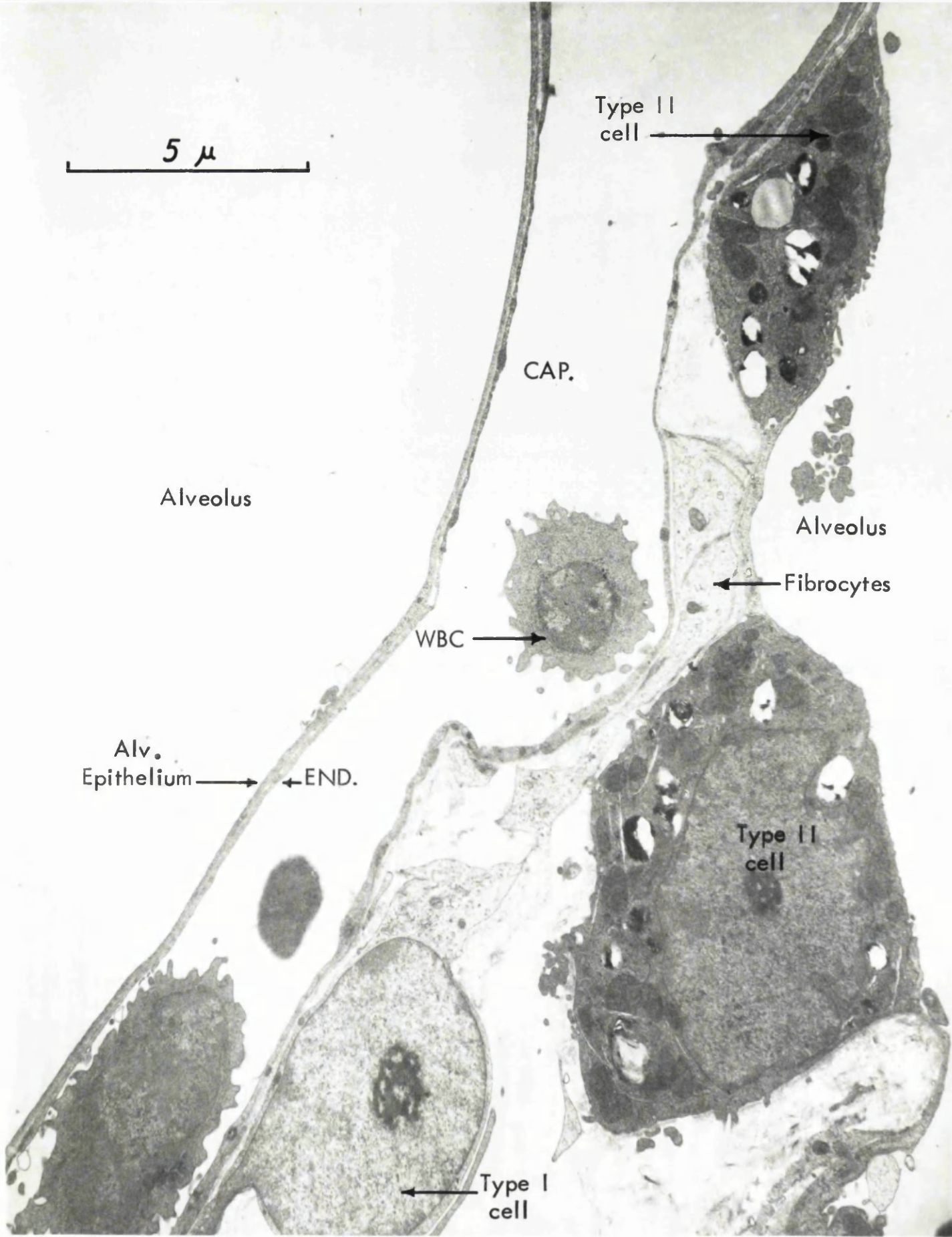


PLATE I - Frontispiece: Montage of the alveolar wall (x 8,250) showing the cell types found in the alveolar wall

CONTENTS

ACKNOWLEDGEMENTS	(i)
SYMBOLS AND SIGNS USED IN THIS STUDY	1

REVIEW OF THE LITERATURE

INTRODUCTION	2
SURFACE FORCES IN INTACT LUNGS.. .. .	4
SURFACE FORCES OF EXTRACTED MATERIAL	6
MORPHOLOGICAL, CYTOCHEMICAL AND BIOCHEMICAL STUDIES	12
MECHANICAL STRESS AND REDUCTION OF PULMONARY SURFACE ACTIVITY	23
PATHOLOGICAL CONDITIONS ASSOCIATED WITH ALTERED PULMONARY SURFACE ACTIVITY	26

PART I

PULMONARY SURFACE ACTIVITY STUDIES

PRESSURE-VOLUME STUDIES

Introduction	36
Methods	39
Results	41
Discussion	44

BUBBLE STABILITY METHOD

Introduction	46
Methods	46
Results	47
Discussion	48

AUTOFLUORESCENCE AND FLUOROCHROME STAINING

Introduction	52
Preliminary/-	

cont.....

Preliminary studies	52
Results	54
Fluorescence of tracheal froth	56
Fluorescence of lung bubbles	57
Effect of hyperventilation	57
Discussion	58

PART II

MORPHOMETRIC STUDIES

INTRODUCTION	62
PRINCIPLES OF MORPHOMETRIC ANALYSIS	
General considerations	65
Measurement of volumes	67
Estimation of surface areas	68
Surface to volume ratios	69
Total number of components	69
Measurement of linear dimensions	70
NORMAL TIDAL VOLUME AND FREQUENCY OF RESPIRATION IN THE GUINEA PIG	72
LUNG VOLUMES AND DEGREE OF INFLATION	76
METHODS OF PREPARATION OF LUNGS FOR MORPHOMETRY	
Introduction	79
Rapid freezing tank-respirator	79
Procedure	81
Freeze substitution fixation	82
Sampling methods	83
CORRECTION FOR SHRINKAGE DUE TO PROCESSING OF THE LUNGS	
Volume shrinkage due to rapid freezing followed by freeze substitution	85
Volume shrinkage due to wax embedding, sectioning and staining	90
PERFUSION FIXATION FOR ELECTRON MICROSCOPY	93

PRACTICAL PROCEDURES USED FOR MORPHOMETRIC ANALYSIS

Measurement of the volume of lung parenchyma	95
Measurement of volumes by point counting	96
Measurement of surface areas by mean chord length	97
Surface to volume ratios	98
Total number of alveoli	98
Measurement of linear dimensions	99

RESULTS OF MORPHOMETRIC ANALYSIS

Volumetric composition	101
Fractional alveolar volumes	101
Total alveolar and alveolar duct volumes	104
Total number of alveoli	107
Alveolar surface area	107
Surface to volume ratios	111
Linear dimensions of alveoli and alveolar ducts	113

ELECTRON MICROSCOPY OF INFLATED AND COLLAPSED LUNGS

Observations	115
Alveolar membrane	117
Air-blood barrier	117

SUMMARY OF THE EFFECTS OF LUNG INFLATION	119
--	-------	-----

SUMMARY OF THE EFFECTS OF HYPERVENTILATION	119
--	-------	-----

DISCUSSION OF THE MORPHOMETRIC ANALYSIS	121
---	-------	-----

GENERAL DISCUSSION	124
--------------------	-------	-----

REFERENCES	126
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APPENDIX I

SIGNS AND SYMBOLS USED IN THIS STUDY

A = Area	a = alveolus
L = Linear chord	d = alveolar duct
S = Surface	f = shrinkage factor
T = Total	l = lung
V = Volume	t = tidal
\underline{V} = Volume fraction	m = mean
cV = Collapsed volume	δa = shape coefficient of alveoli
S_{\downarrow} = Shrinkage	φ = Volume fraction of the lung parenchyma.

e.g. Total alveolar surface area	TS_a
Volume fraction of alveoli	\underline{V}_a
Total volume of lung parenchyma	$\varphi \cdot TV_L$
Mean chord length	L_m

REVIEW OF THE LITERATURE

INTRODUCTION

The lungs have been known to have a complex internal structure since they were first described in detail by Malpighi in 1661. He recognised the 'vesiculae' as the endings of the airway system and that they were separated from each other by a thin membrane containing the 'rete mirabile'.

In 1731 that enthusiastic researcher from rural England, the Rev. Stephen Hales attempted to correlate structure and function of the membrane when he wrote that the blood is 'by an admirable contrivance there spread into a great expanse, commensurate to a very large surface of air, from which it is parted by very thin partitions; so very thin, as thereby probably to admit the blood and air particles within reach of each others' attraction'. The function of the internal pulmonary surface in relation to gas exchange has been thoroughly investigated since this original interpretation and is now well established.

The possibility that the alveolar surface membrane, might have functions and properties other than those of gas exchange, was not alluded to until just forty years ago, when the Swiss physician von Neergaard, (1929) found that the retractive force of an air-filled lung was greater than that of a lung filled with fluid. He suggested that surface tension within the lungs accounted for this difference.

It was pointed out in 1957 by Radford that if the surface tension of the lungs followed the La Place relationship (see p. 5) it would tend to accelerate collapse of the lung as total lung volume is reduced. Normally the lungs do not collapse completely because of the specialised nature of the lung

surface tension forces which differ from those of other biological and organic systems in that they have very much lower values (Radford, 1957). Thus the tendency to complete collapse is lessened because surface forces are low at low lung volumes.

This implies that the internal surface membrane is by itself unique or that it is lined by a substance which has the unique property of a very low surface tension. The latter view is the one currently favoured although the former cannot be dismissed entirely (Hughes, May & Widdicombe, 1959). More recent work, however, indicates that the mechanical properties of the alveolar tissue as distinct from its surface, are similar to many other biological materials (Fukaya, Martin, Young & Katsura, 1968).

Since the fundamental property of this lining 'substance' is that its surface tension is low, Clements (1962) has called it 'Pulmonary Surfactant'. It has been variously described as 'alveolar lining layer' (Pattle, 1955), 'alveolar lining complex' (Pattle, 1958), 'lung surfactant' (Buckingham & Avery, 1962) and 'surface active lipoprotein' (Abrams & Taylor, 1964; Taylor & Abrams, 1964, 1966; Abrams, 1966). Pulmonary surfactant is the term now most widely used and is to be preferred. (Avery & Said, 1965).

The evidence for the existence of a specific substance to which one can ascribe the name, pulmonary surfactant, and the unique properties of the internal lung surface will now be discussed.

Recent studies, which have included many comprehensive reviews (Mead, 1961 & 1962; Avery, 1962; Clements, 1962; Pattle, 1961; 1963 & 1965; Wyss, 1963; Avery & Said, 1965; Clements & Tierney, 1965; Scarpelli, 1967), encompass a great diversity of scientific discipline so that it is perhaps advantageous for the sake of clarity to deal with each discipline separately where possible.

SURFACE FORCES IN INTACT LUNGS

Since Von Neergaard's original observations (see page 37) the most informative studies have been those which used the relatively simple manoeuvre of obtaining pressure-volume relationships for excised lungs.

Lungs of new born animals:

The importance of surface tension in determining the uniformity of lung expansion at birth is indicated by the finding that in excised lungs of stillborn infants whose lungs were normal in appearance, the pressure required to inflate the lung was greater with air than with fluid (Gruenwald, 1947).

Air filled and fluid filled lungs:

Air filled lungs show marked hysteresis between the pressure volume curve during inflation and during deflation, while in fluid filled lungs where surface forces are minimised the hysteresis is slight, (Mead, Whittenberger & Radford, 1957).

The effect of detergents:

The role of surface forces can be evaluated by changing them with detergents, which will interact with the surface but not the underlying tissue, and comparing the pressure-volume relationships of detergent treated and normal lungs. Filling the lungs with saline then emptying them and inflating with air does not alter the normal pressure volume relationship (Radford, 1964). However, the introduction and subsequent removal of 0.5% polysorbate (Tween 20) followed by air filling resulted in marked alteration of the pressure volume relationship. Detergent has a high surface tension which resulted in early collapse of the lungs, at a transpulmonary pressure of 8 cms.H₂O. Normally at a similar pressure the lungs are about two thirds inflated.

The pressure required to inflate airless lungs can be reduced by the presence of detergents such as amyl acetate (Gruenwald, 1947), 0.5% octyl alcohol in plasma, or petroleum ether (Yoshida, 1962). However the major effect of such substances is on the deflating part of the respiratory cycle by causing premature collapse (Pattle & Burgess, 1961).

Detergents are thought to act on the alveolar surface and not on the tissue itself, because they do not affect the length tension properties of strips of lung tissue (Yoshida, 1962). Dog lungs filled with mercury, which has a high and probably fairly constant interfacial tension with the lung interior, retain only 7% of the maximum volume of mercury when allowed to deflate (Pierce, Hocott & Hefley, 1961).

THEORETICAL CONSIDERATIONS

It has been said of surface tension that 'the molecules at the surface are attracted more strongly to their neighbours below the surface and are attracted only weakly to the sparcer population of molecules in the air above the surface. Because the net pull is downward, the surface molecules tend to dive and the surface shrinks to the least possible area', (Clements, 1962).

The relationship between surface tension which is force per unit distance (in dynes/cm.) and the pressure within a bubble suspended in a liquid is given by the La Place equation, where for spherical bodies the pressure equals the ratio of twice the surface tension to the radius of curvature; $P = 2\gamma/r$. Since the lungs have a very large though admittedly complex internal surface and since the radius of curvature of the normal population of alveoli is small by comparison, it is obvious that surface tension in the lungs must be functionally important, (Avery & Said, 1965).

The conditions under which surface tension will cause the lungs to

collapse have been theorised by the construction of mathematical models. The most useful and elaborate is that devised by Clements, Husted, Johnson & Gribetz (1961). They likened the alveolus to a bubble blown on the end of a tube and calculated the condition that an alveolus will remain stable, in terms of its radius, surface tension, and surface elastance when the pressure exceeds a certain minimum. The equation they derived is $P = (8\gamma - 4s)/3r$ where the minimum pressure is P , surface tension is γ , the radius is r and surface elastance is s which is defined as the change in tension with area and equals $A \delta \gamma / \delta A$. Thus stability of the alveolus model is favoured by high internal pressure, large radius, low surface tension and high surface elastance. Measurements of surface tension of material extracted from the lungs has tended to prove the validity of this concept.

SURFACE FORCES OF EXTRACTED MATERIAL

STABLE LUNG BUBBLES

Observations: The foam of acute pulmonary oedema is very stable, with a consistency of whipped egg albumin: this observation is certainly not new. (Avery & Said, 1965). Other body fluids such as plasma and synovial fluid can be made to foam but do not have the same stability.

Serum has a surface tension of 55 dynes/cm. (Du Nouy, 1926) and if surface tension in the lung was normally at this level a much greater pressure would be required to keep the lung inflated than is the case (Pattle, 1965).

The significance of the stability of lung foam was highlighted by studies on its resistance to chemical antifoam agents such as silicon antifoam and octyl alcohol (Pattle, 1956 & 1958) which had previously been reported as having therapeutic value in the treatment of acute pulmonary oedema (Curry & Nickerson,

1952; Nickerson & Curry, 1955).

These antifoams rapidly and completely destroy the foams of blood and oedema fluid from sites other than the lungs. The resistance to them by lung foam is dependent on a low surface tension and high surface activity of the foam (Pattle, 1950). This was shown by observing bubbles of lung foam under the microscope: bubbles as small as 1μ in diameter remained stable for more than twenty minutes. A bubble in air-saturated blood or water contracts and disappears within a few minutes because surface tension inside the bubble causes the pressure inside to be greater than atmospheric pressure and the gas within the bubble diffuses out into the solution (Pattle, 1955). This phenomenon has been analysed mathematically for bubbles having an internal pressure slightly above atmospheric pressure and having a constant surface tension. The lifetime of such bubbles is approximately proportional to the cube of the initial diameter (Epstein & Plesset, 1950; Fox & Herzfeld, 1954; Pattle, 1960).

Possible explanation:

The stability of lung bubbles might be due to the presence of a lining film impervious to gaseous diffusion (Clements, 1957). This is disputed by Pattle (1960) who found no evidence of impermeability to gaseous diffusion and who suggests that these bubbles probably have a very low surface tension in air-saturated water perhaps as low as 0.1 dynes/cm. For most organic liquids the surface tension is around 30 dynes/cm.

Pattle (1965) has also demonstrated that the froth from the lung can be washed by shaking it in distilled water without altering the stability of bubbles subsequently obtained from it. When the bubbles were dissolved in air free water they left behind a 'transparent, irregularly shaped ghost' of the bubble indicating that the lining of the bubble was at least partly insoluble in water.

Recently it has been shown that an insoluble lamellar precipitate can be produced from the clear supernatant of centrifuged surfactant when it is treated with calcium chloride, (Mendenhall, Sun & Mendenhall, 1967). This precipitate is thought to be indicative of marked interfacial turbulence due to Thompson Marangoni effects which might also explain the appearance of the precipitate in air free water which Pattle observed (Mendenhall, Mendenhall & Tucker, 1967).

SURFACE TENSION AND SURFACE AREA

Surface forces at high lung volumes account for 75% of lung elasticity; at low lung volumes they account for only 30% of lung elasticity. These findings can be reconciled if one considers that the surface tension in the lungs changes as the internal surface area changes, (Clements, 1957).

In vitro surface balance studies:

It has been known for some time that the surface tension of a fluid could change if the surface area was changed, (Pockles, 1891). Clements used a modified Wilhelmy surface tension balance to measure the surface tension of extracts of minced lung. The minced lung was washed in 0.9% saline and filtered through gauze. It was then resuspended in fresh saline and placed in a shallow Teflon tray. A platinum strip, 0.001 inches thick, was suspended in the fluid from the arm of a strain gauge. A barrier placed across one end of the tray was moved in and out cyclically, reducing the surface area of the fluid in the tray then expanding it to its original size, (Fig. 11).

Clements demonstrated that surface tension increased when the surface area was increased and decreased when the surface area was reduced, (Clements, Brown & Johnson, 1958). There was also marked hysteresis between the surface tensions obtained when the surface area was being expanded and

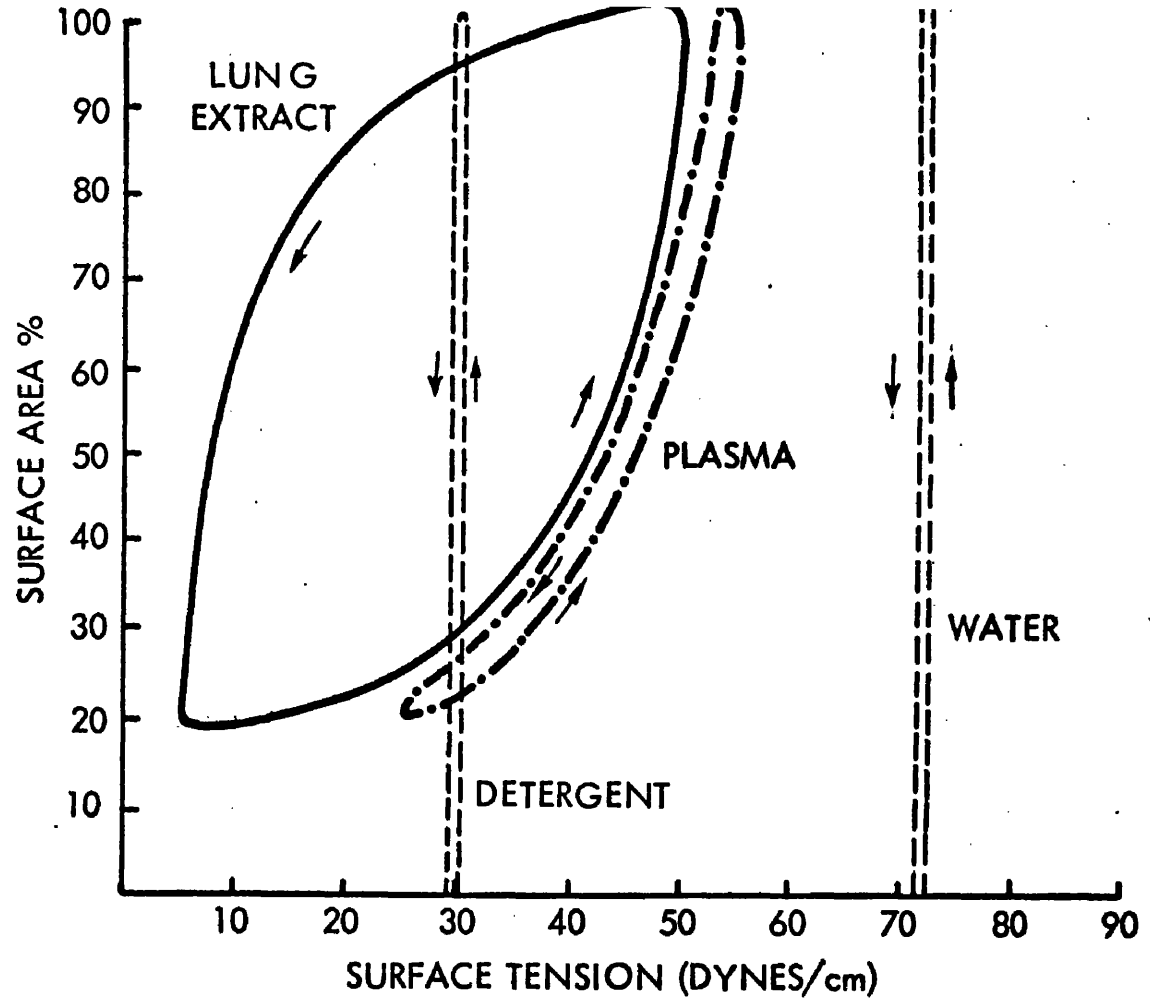


Fig. I - SURFACE AREA - SURFACE TENSION LOOPS

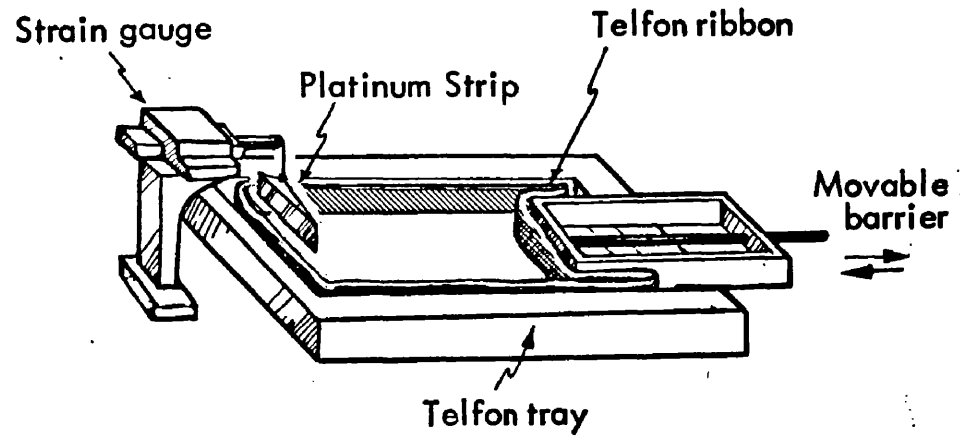


Fig. II - MODIFIED WILHELMY SURFACE BALANCE FOR measuring surface area - surface tension relationships

when it was being reduced. This would indicate that the surface of the fluid possessed inherent elasticity. The values for the minimum surface tension obtained by this method lay between 5 and 10 dynes/cm. (Fig. 1).

Recently the role of pulmonary surfactant in lung elasticity and stability has been questioned, (Mendenhall & Mendenhall, 1964). They found that there was virtually no elasticity of the surface film of lung washings on the surface tension balance when measurements were made under static conditions.

There is little change in the retractive pressure of the intact lung when kept inflated for up to forty minutes; but when the cyclical alteration of surface area on the surface tension balance is stopped during the measurement of surface tension, the surface tension abruptly moves towards an equilibrium value of 20 - 27.5 dynes/cm. The same equilibrium tension results whether the barrier is stopped when reducing the surface area or increasing it. If excess fluid was present in the Teflon tray of the surface tension balance the equilibrium tension was slightly lower. They suggest that in either case the tension is too great for pulmonary surfactant to be the main factor in alveolar stability, although the surface tension is less and the stability greater than that which would be provided by a surface of pure water. Similar findings have been noted by others, (Sutnick & Soloff, 1963).

The relevance of these observations to the normal intact lung is speculative: They question the validity of relating pressure-volume relationships of intact lungs to the surface area - surface tension relationships of extracted material: it has to be recognised that different physical forces may be responsible for the particular characteristics in the two situations.

Methods of obtaining surface active lung material:

There are almost as many methods of obtaining surface active lung material as there are workers studying it. The most commonly used are

1. mincing pieces of lung tissue and subsequently washing in saline and filtering through gauze, (Clements, 1957),
2. homogenisation of lung slices, (Reiss, 1965),
3. washing saline down the trachea and compressing the lungs in saline, (Avery & Mead, 1959),
4. collection of the tracheal effluent during perfusion of the pulmonary vascular bed with saline, (Bondurant & Miller, 1962).

The site of origin of the surface active solutions obtained is difficult to define in all these methods. The case for the alveolar surface being the site of origin has been thoroughly argued, (Pattle, 1958); as yet there is no conclusive proof that this is so. It is also of interest that the values for surface tension obtained by the different methods differ.

pH and temperature effects:

Several workers have tested the possibility that pH and temperature might affect the surface tension properties of extracted lung material. The results of these studies are conflicting.

Avery and Mead (1959) found that changing the pH from 1 - 11, or changing the ambient temperature from 70 - 101°F, of the fluid extracted from human lungs, had no effect on the surface tension measured on a modified Wilhelmy surface tension balance, Radford (1964) changed the temperature of excised lungs from 20 - 37°C and found that it did not alter their elastic behaviour.

On the other hand, an increase in the minimum surface tension measured has been shown to occur when the lung extract in the surface balance was heated from 25 - 45°C. This effect was reversed on recooling, (Tierney &

Johnson, 1961 & 1963). Heating the intact lungs of rats causes premature collapse during pressure-volume manoeuvres, (Clements, Tierney & Trahan, 1963; Clements & Trahan, 1963).

An increase in temperature from 37 - 48°C of newborn human and rat lungs stabilises abnormal lungs and promotes collapse in normal lungs so that the surface properties at the higher temperature are similar, (Gruenwald, 1964).

Potassium hydroxide extracts of dog lungs at pH 8.5 have been shown to be less surface active than saline extracts at pH 6.4, (Sutnick & Soloff, 1964).

Summary of the surface activity of intact lungs and extracted fluid:

The important features concerning the surface activity of intact lungs and the surface tension of extracted lung material may be summarised as follows:-

1. Surface tension accounts for most of the hysteresis seen in pressure-volume relationships of intact lungs.
2. Bubbles expressed from lungs are stable.
3. The surface tension of extracted pulmonary surfactant is low at reduced surface area, and high at increased surface area when the surface area is changed cyclically.
4. When the cyclical alteration of surface area of the fluid in the surface tension balance is halted during measurement of surface tension, the surface tension rapidly reaches an equilibrium value. This equilibrium tension lies between the minimum and maximum surface tensions recorded when the surface area is continuously and cyclically changed.

Difficulties arise when one attempts to correlate the findings of studies on the surface forces in the intact lung with those on the surface tension properties of extracted lung material. This is because there is no way of measuring the surface tension in the intact lung.

Despite these difficulties the following theory is widely held to be the most likely explanation of the role of pulmonary surfactant:-

The alveoli are lined by a substance which lowers surface tension as the alveolar surface area is reduced. This results in a lower pressure required to keep the alveolus patent so that the possibility of complete collapse is lessened.

This theory rests on two assumptions, neither of which has been thoroughly investigated until now. Firstly, that the alveolar surface area changes with a change in lung volume and secondly, that it changes sufficiently to effect a change in the surface tension of the alveolar surface.

Other functions attributed to pulmonary surfactant:

Recently it has been suggested that the uptake of certain gases in the lung is dependent on the presence of pulmonary surfactant at the alveolar surface, (Ecanow, Balagot & Santelices, 1967).

Non-polar gases such as the anaesthetic gas halothane are not very soluble in water or saline; the presence of surfactant made halothane readily soluble in both these fluids. This would indicate that surfactant at the diffusing membrane of the alveolus would greatly facilitate the transfer of non-polar gases across the alveolar epithelium.

Similar findings have been reported by others for ether as well as halothane, (Miller & Thomas, 1967).

MORPHOLOGICAL AND CYTOCHEMICAL AND BIOCHEMICAL STUDIES

Great advances have been made in the morphology of the alveolus since the introduction of the electron-microscope as a research tool. The cellular continuity of the alveolar surface has been proved in rat lungs, (Low & Daniels, 1952). They demonstrated an alveolar lining cell overlying the capillary endothelium. The thickness of this cell varied between 0.1 - 0.7 μ ,

and the nucleus was flattened; this led them to the conclusion that these cells were endodermal in origin. Shortly after this work, cellular continuity between the cuboidal bronchiolar cells and these attenuated alveolar lining cells was demonstrated, (Low & Sampaio, 1957).

Cell types in the alveolar epithelium:

There are now recognised to be three distinct cell types making up the alveolar lining, (Macklin, 1950 & 1954).

1. Extremely flattened cells, visible only with the electron-microscope, known as membranous pneumonocytes or type I cells, also as squamous alveolar cells, small alveolar cells, and pulmonary alveolar cells.
2. Approximately cuboidal cells which do not extend their cytoplasm beyond the perikaryon, and which are of sufficient thickness to make them visible under the light microscope. These cells are known as granular pneumonocytes or type II cells, also as great alveolar cells, large alveolar cells, and septal cells.
3. Pleomorphic cells usually loosely attached to the alveolar wall and separated from the basement membrane of the capillary endothelium by the cytoplasmic extensions of the type I cell. These cells are alveolar phagocytes or dust cells.

The other cells which make up the wall of the alveolus are mesenchymal connective tissue cells, mainly fibrocytes, and the endothelial cells of the alveolar capillaries.

Type II cells:

Most interest in the cells of the alveolus has centered on the granular pneumonocyte or type II cells, particularly since it was suggested that they have an exocrine function, (Macklin, 1954), and are responsible for the production of a 'semiliquid lining layer' on the alveolar surface. Macklin considered the

'secretion' of these cells to be mucoid on the basis of their affinity for colloidal iron.

Light microscopic appearance:

Typically the type II cells are found at the corners where adjacent alveoli meet, (Bertalanffy, 1964). They have an irregularly cuboidal shape, a vacuolated cytoplasm and a large vesicular nucleus.

On paraffin sections, stained with pairs of acidic and basic dyes, the cytoplasm is moderately basophilic and the vacuoles are unstained. On frozen sections, there is often difficulty in distinguishing them from alveolar phagocytes, (Sorokin, 1967).

Cytochemical studies on the type II cell:

The cytochemical properties of the type II cell are not well known because of the difficulty of identifying them on paraffin and frozen sections. Nonetheless they are stained specifically on frozen sections with Sudan Black B, and on Epon embedded sections by the PAS method. They can be distinguished from alveolar phagocytes because they are less reactive for succinic dehydrogenase and acid phosphatase, (Sorokin, 1967).

It has been suggested that the vacuoles in the cytoplasm of the type II cell might be transformed mitochondria, (Schulz, 1959), but this seems unlikely because of the absence of acid phosphatase activity and the presence of alkaline phosphatase activity, (Buckingham, McNary & Sommers, 1964).

The mitochondria of the type II cell have been frequently suggested as being a possible site of pulmonary surfactant synthesis, (Klaus, Reiss, Tooley, Piel & Clements, 1962; Reiss, 1962, 1965, 1966 & 1967; Hackney, Bils, Takahashi, Rounds & Collier, 1967). It seems likely that the mitochondria, which are usually abundantly distributed in the type II cell cytoplasm, take part in the 'secretion' of this cell, (Bensch, Schaefer & Avery, 1964).

ELECTRONMICROSCOPIC STUDIES ON THE TYPE II CELL

The type II cell is characterised by multilamellar inclusion bodies in the cytoplasm which also has well developed Golgi and endoplasmic reticular systems. It has frequently been described by electronmicroscopists*.

Evidence for the epithelial nature of the type II cell:

The epithelial nature of the type II cell is fairly well established, despite the fact that unlike most other epithelial cells it is markedly pleomorphic, within the limits of an approximately cuboidal shape, (Bertalanffy & Leblond, 1955; Low & Sampaio, 1957; Policard, 1959; Sorokin, 1967), since it was shown that these cells rest directly on the basal lamina of the alveolar epithelium. Further evidence that they are of epithelial origin is given by

1. they exhibit polarity in that their free surfaces have microvilli,
2. they form junctional complexes with the attenuated processes of the type I cells, and
3. it has been noted in foetal lungs that lamellated inclusion bodies similar to those seen in the mature type II cell, are found only in the cuboidal epithelial cells that line the distal ramifications of the bronchial tree, (Sorokin, 1967).

Comparative studies:

The lungs of all mammalian species studied so far have type II cells or cells very similar to them in the alveoli. Similar cells have been shown to be present in the primitive lungs of amphibians, (Okada, Ishiko, Daido, Kim &

*Bertalanffy & Leblond, 1955; Collet, Basset & Normand-Reuet, 1967; Campiche, 1960; Dreissens, Dupont & Demaille, 1959; Engel, 1962; De Groot, Lagasse & Sebruyns, 1958; Von Hayek, 1953 & 1960; Karrer, 1956a, 1956b, 1956c, 1958 & 1960; Kisch, 1955; Krahl, 1959; Low & Daniels, 1952; Policard, 1956, 1957a, 1957b & 1959; Policard, Collet & Pregermain, 1956; Schlipkoter, 1954; Schulz, 1959 & 1962; Sorokin, 1967.

Ikeda, 1962a; Sorokin, 1967) and of reptiles, (Okada et al, 1962b).

Evidence for the secretory function of the type II cell:

Most of the morphological evidence suggests that the type II cell functions primarily by synthesising and secreting cytosomal material onto the cell surface. Sorokin (1967) has postulated the steps leading up to its extrusion from the cell; beginning with the coalescence of minute multivesicular bodies to the formation of cytosomes which later become lamellated with osmiophilic micelles and enlarge, and which are finally released onto the cell surface by breakdown of the plasma membrane which surrounds them. He demonstrated that the cytosomes contain large amounts of lipid material; mainly phospholipid and triglyceride. Much of the osmiophilia which is characteristic of these cytosomes has been shown to be due to unsaturated lipids, (Bahr, 1954; Hayes, Lindgren & Gofman, 1963). They may also contain phosphatides and polysaccharides, (Sorokin, 1967) and protein, (Baylis & Conen, 1964). Pulmonary phospholipid synthesis occurs predominantly in the type II cell, (Harlan, Said, Spiers, Banerjee & Avery, 1964; Buckingham, Heinemann, Sommers & McNary, 1966).

Evidence that the type II cell secretes pulmonary surfactant:

Cytosomes have been regarded for some time as possible stores of pulmonary surfactant, (Campiche, Gautier, Hernandez & Reymond, 1963; Buckingham, McNary & Sommers, 1964) which upon release from the type II cell form a coating on the alveolar surface, thereby conferring on the alveolus a surface tension lowering effect, (Pattle, 1958; Clements, Brown & Johnson, 1958).

While implicated in this activity as a potential source of phospholipids capable of exerting this effect, (Klaus, Clements & Havel, 1961; Klaus, Reiss

et al, 1962), the cytosomal material nonetheless has not been shown to be identical with pulmonary surfactant. Indeed the agent known to be largely responsible for the surface tension lowering effects of pulmonary surfactant is the saturated phospholipid, dipalmitoyl lecithin, (Clements, 1966) which is not osmiophilic, unlike the cytosomes of the type II cell.

The appearance of cytosomes, however, can be modified easily by altering the carbon dioxide tension of inspired air, (Bensch, Schaefer & Avery, 1964) and by hypoxia, (Valdavia, Sonnad & D'Amato, 1966). Variation of the dietary lipids can also alter the appearance of stored phospholipid, (De Gier & Van Deenan, 1964); so that the postulate may still be valid, that these cytosomes in the type II cell are stores of pulmonary surfactant. There has been the suggestion that pulmonary surfactant is produced by the non-ciliated bronchiolar cells (Clara cells) lining the terminal and respiratory bronchioles, (Niden, 1967); there is a slow drift of pulmonary surfactant from these terminal airways into the alveoli where it is eventually phagocytosed by the alveolar macrophages.

Evidence for a phagocytic function of the type II cell:

When Thorotrast, (Low & Sampaio, 1957) or Indian ink, (Karrer, 1958) is introduced down the airways into the lungs, it is taken up by the alveolar phagocytes. When type II cells are cultured on a tissue plate they ingest Thorotrast particles. These are easily seen in the cisternae of the endoplasmic reticulum and to a lesser extent in the cytosomes, (Ladman & Finley, 1966). This suggests that the type II cell may possess a limited capacity for phagocytosis, so that Niden's hypothesis of downstream movement of pulmonary surfactant is not disproved, if one accepts that the type II cells may be primarily phagocytic and not secretory. The difficulty in identifying the source of pulmonary

surfactant lies in the fact that all histological observations are static and not easily related to the dynamic process.

Further evidence that type II cells secrete pulmonary surfactant from studies in foetal lungs:

Evidence that the cytosomes of the type II cell are associated with pulmonary surfactant is given by studies done on various mammals during foetal life.

Stable lung bubbles are first formed in the lungs of mice at about seventeen days gestation, (Pattle, 1958). The ability to produce a low surface tension on the surface tension balance by extracted mouse lung material occurs at eighteen days gestation, (Buckingham & Avery, 1962).

Osmiophilic inclusion bodies first appear in the type II cells of mouse lungs at the same time, (Woodside & Dalton, 1958). In humans the time of appearance of inclusion bodies is about the fifth or sixth month of intrauterine life, (Campiche, Jaccottet & Juillard, 1962; Campiche, Gautier, Hernandez & Reymond, 1963). The typical surface tension properties of the mature lung appears at a foetal body weight of 1 - 1.2 kg., (Avery & Mead, 1959) which is consistent with a five or six month foetus; although the difficulty of obtaining adequate specimens complicated this correlation study.

In lambs which were asphyxiated and premature at birth and whose lungs showed evidence of reduced surface activity, there was a reduction in the number of inclusion bodies in the type II cells, (Orzalesi, Motoyama, Jacobson, Kikkawa, Reynolds & Cook, 1965).

The above findings would indicate that there is more than a passing relationship between pulmonary surfactant and the type II cell; but it is not possible to define exactly what that relationship is, so that the origin of pulmonary surfactant is still unsolved.

Electronmicroscopy of the alveolar surface of the type I lining cell:

Several workers have described a thin electron-dense layer at the alveolar surface. Chase (1959) found a thin granular layer at the alveolar surface of freeze dried sections of the lungs of rats, mice, bats and one chicken, but not in the lungs of frogs. The granular layer was not seen when the lungs were fixed with buffered osmium tetroxide or when there was evidence of pneumonic exudate.

Groniowski and Biczyskova (1964) found an irregular layer of compact granules on the alveolar surface of three rabbit lungs fixed in 0.5% potassium permanganate buffered to pH 7.4 in veronal acetate, and embedded in Araldite D (Ciba). The thickness of the layer of granules varied between 200 - 1000 Å. They also found that the granules were present in the cytoplasm of the type I attenuated lining cells. On staining the sections with a modified Hale staining method; first described by Gasic & Berwick (1963), and whose basis is colloidal iron, specific for mucopolysaccharides, Groniowski and Biczyskova demonstrated a layer of some 300 Å thickness. This Hale positive layer was continuous over the alveolar surface including the alveolar phagocytes; although there was no Hale positive material within the cytoplasm of the type I cell.

Klika and Janout (1967) using a different staining method; Maillets' modification of Champy's method, were able to demonstrate a similar lining layer at the alveolar surface. Petrik and Riedel (1968) found a continuous osmiophilic layer which was non cellular, on the alveolar surface of foetal chick lungs and the lungs of three day old chickens.

The alveolar surface has recently been shown to be lined with a membranous structure, (Harrison & Weibel, 1969) visible on the electronmicroscope as electron dense wavy lamellated lines outside the plasma membrane of the type I attenuated lining cell. These lamella had been thought earlier to be a duplex

layer, (Weibel & Gil, 1968) but it is apparent that the structure is more complex. The thickness of the membranous layer varied greatly. It was thinnest over the flattened portions of the alveolar wall and thickest at the corners between adjacent alveoli. These workers suggest that this structure is pulmonary surfactant lining the alveoli, however much more work would seem to be required before such a definitive statement can be made.

BIOCHEMICAL STUDIES OF EXTRACTED PULMONARY SURFACTANT

Klaus, Clements & Havel (1961) were the first to analyse lung extracts and relate their contents to surface activity. They found that the phospholipid fraction reduced surface tension by as much as the crude extract. They also showed that purified lysolecithin, sphingomyelin from red blood cells, and synthetic dipalmitoyl lecithin all had similar surface tension properties to the crude lung extract. More recently purified phospholipids and synthetic dipalmitoyl lecithin have been further studied and shown to have essentially similar surface tension properties to pulmonary surfactant, (Watkins, 1968; Galdston & Shah, 1967).

The most surface active component of pulmonary surfactant is phosphatidyl choline, (Fujiwara & Adams, 1964; Fujiwara, 1965; Pattle & Thomas, 1961); although it has been suggested that dipalmitoyl lecithin is the main constituent responsible for the surface tension lowering effect of pulmonary surfactant, (Brown, 1964).

Studies on the molecular structure of pulmonary surfactant:

It is not clear whether the surface active phospholipids are free moving molecules in the alveolar lining or present as combinations with other molecules. It has been suggested that they are associated with other lipids or carbohydrates, (Scarpelli, Clutario & Taylor, 1967). Pulmonary surfactant has frequently been

described as a lipoprotein, (Avery & Said, 1965; Brown, 1964; Abrams & Taylor, 1964; Reynolds & Strang, 1966) but there is little evidence that this is so. The association with a protein moiety is positively disputed by Scarpelli et al (1967), however there is evidence that alveolar cells produce protein in significant amounts, (Massaro, 1967).

In only two studies has a lipoprotein been identified in lung extracts, (Abrams & Taylor, 1964; Abrams, 1966). A protein of similar electrophoretic mobility as α globulin was found to be 'highly surface active'. Other evidence that pulmonary surfactant is a lipoprotein is indirect.

Pattle & Thomas (1961) found that tracheal washings had the same infra red absorption spectrum as a mixture of 5% gelatin and 95% egg lecithin. Several workers have reported that proteolytic enzymes reduce the surface activity of lung extracts, (Pattle, 1958; Tierney & Johnson, 1963; Johnson, Levine & Cummings, 1964). Boland & Klaus (1964) however, found that digestion of lung extracts by trypsin did not alter the surface activity.

Fibres derived from the surface films of extracts of sheep lungs contain 14.5% nitrogenous material and stain similarly to thromboplastin, (Buckingham, 1961). When a solution of extracted pulmonary surfactant is mixed with trichloroacetic acid, complete precipitation occurs, indicating a significant protein fraction, (Brown, 1964).

The question of contamination of lung extracts with plasma protein has been investigated, (Scarpelli et al, 1967). When a pure sample of lung extract is fractionated no protein is present in the surface active fraction. Analysis of this fraction yielded a mixture of phospholipids; mainly lysolecithin, sphingomyelin, neutral lipids, phosphatidyl ethanolamine and phosphatidyl choline, and a complex mixture of polysaccharides. All the lipids were surface active with the exception of phosphatidyl ethanolamine. This confirmed earlier findings by others, (Brown, 1964; Fujiwara & Adams, 1964; Klaus, Clements & Havel, 1961). Scarpelli et al (1967) also found that in one experiment where

blood contamination of the lung extract was marked, a protein was recovered on fractionation. This protein was similar to β lipoprotein normally found in serum. Its surface activity was similar to pulmonary surfactant. They suggest that there may be a blood surfactant system, the function of which however is obscure.

Staining reactions and fluorescence of the alveolar lining:

Since lipids fluoresce, (Udenfriend, 1962), and since pulmonary surfactant contains lecithins, (Klaus, Clements & Havel, 1963) it was not long before work was begun on the fluorescent properties of pulmonary alveoli, (Hackney, Rounds & Schoen, 1963). Hackney, Collier & Rounds (1965) produced stable bubbles when frozen sections of unfixed lung were allowed to thaw. These bubbles showed bright fluorescence which was selectively quenched by short exposure to osmic acid and iodine vapour fumes. When the bubbles were allowed to dry they were stained by the fluorochrome dyes, protoporphyrin, rhodamine B and 6G, phosphine GN and with Sudan 111. In all cases the dye was taken up by the bubbles.

A bright autofluorescent yellow line was seen lining the alveoli on frozen unfixed sections. Reduced alveolar fluorescence was seen in animals subjected to oxygen poisoning, furfuralacetone poisoning or bilateral vagotomy, and in human infants who had died of acute respiratory distress syndrome. This correlated with a reduction of the surface activity of extracted material as well as reduction of the stability of expressed lung bubbles.

Fluorescent lines at the alveolar surface have been demonstrated in calcium formalin fixed lung sections, mounted in glycerine, (Boland & Klaus, 1964; De Sa, 1965). De Sa, was able to show also that on sections stained with acid haematin; for phospholipids, and with phosphomolybdic acid; for choline containing lipids, the dyes took at exactly the same place on the alveolar surface as fluorochrome dyes. The distribution of dye reaction tended to be more prominent at the corners of adjacent alveoli.

MECHANICAL STRESS AND REDUCTION OF PULMONARY SURFACE ACTIVITY

MECHANICAL STRESS

Schulz (1959) first suggested that mechanical stress of the alveolar lining could change its surface properties. He found that overinflation causes granular cytoplasmic degeneration and disruption of the alveolar cells.

Studies on extracted pulmonary surfactant:

The surface tension of extracted pulmonary surfactant falls progressively when the surface area of the film in the surface tension balance is not altered, (Clements, Brown & Johnson, 1958). Expansion of the surface area reverses this process causing the surface tension to return to the initial value.

If the surface film is compressed to not less than 50% of the initial surface area, normal surface activity is maintained, (Brown, Johnson & Clements, 1959); but compression to more than 50% of the initial surface area, followed by re-expansion causes rupture of the surface film. This implies that compression to such a degree results in irreversable solidification or folding of the surface molecules. Slight compression of protein films causes rapid solidification with rupture on re-expansion, (Hober, 1945); this suggests that pulmonary surfactant lacks a protein moiety or, if present, is atypical.

Repeated expansion and compression of the surface area of extracted pulmonary surfactant causes those constituents which are particularly surface active to concentrate at the surface, (Tierney & Johnson, 1965). They suggest that when there is disruption of the surface molecules, fresh surfactant is formed by migration of surface active material from the hypophase. This implies that a substantial hypophase layer of potential surfactant exists in the alveolar lining.

Studies on intact lungs:

Compliance is often used as a measure of elastic or retractive forces in the lung. It is defined as the ratio of the change in lung volume to the change in retractive pressure, expressed as litres/cm.H₂O

In anaesthetized dogs, compliance falls progressively whether they are breathing spontaneously, or are paralysed with succinylcholine and ventilated artificially within a resting tidal volume range, (Mead & Collier, 1959). Occasional forced inflations up to vital capacity level causes the compliance to return to normal values, whereas forced deflations causes a further fall in compliance. The lungs which had the greatest fall in compliance consistently showed histological evidence of widespread atelectasis. This confirmed earlier observations, (Bernstein, 1957) that in anaesthetized rabbits, periodic forced lung inflations increased compliance; between the periodic inflations compliance fell steadily. This has been suggested as the possible functional significance of sighing, (Clements & Tierney, 1965).

Intermittent positive pressure ventilation:

Intermittent positive pressure ventilation (IPPV) during prolonged surgical anaesthesia causes a reduction in lung compliance, (Holaday & Israel, 1955; Brownlee & Albritten, 1956). The time course of the changes in compliance are not described in either of these studies. Wu, Miller & Luhn, (1956) measured compliance on surgical patients before and after anaesthesia, in the supine position. They found that two hours after induction of anaesthesia, compliance had fallen to 65% of the pre-anaesthetic value, but thereafter, showed little change.

Lungs which are filled with fluid and subsequently artificially ventilated, have a low compliance, and their extracts consistently have high surface tensions,

(Johnson, Permutt, Sipple & Salem, 1964). Prolonged IPPV, with hyperinflation, causes 'depletion, alteration or interference with the action of pulmonary surfactant', (Greenfield, Ebert & Benson, 1964).

Effects of ventilation rate and volume on lung surface forces:

Ventilation of excised dog lungs increased the surface forces, i.e. reduced lung compliance, by an amount which is directly related to the tidal volume and the duration of ventilation and is inversely related to the end expiratory pressure, (Faridy, Permutt & Riley, 1966). Ventilation with 100% nitrogen caused a more marked fall in compliance than with 100% oxygen or air.

If a lung is kept inflated but not ventilated there is no change in compliance, (Permutt, 1967); but ventilation causes a fall in compliance. If after compliance has fallen the lungs are kept inflated at constant volume, at room temperature and in oxygen, compliance rises to the pre-ventilation value. This implies that the recovery of surface activity in the lungs after it has been reduced by ventilation depends on metabolic processes.

It has been suggested that ventilation causes a reduction in the amount of effective pulmonary surfactant on the alveolar surface; but provided that the cells producing new surfactant are functionally normal, return of normal surface activity quickly occurs, (McClenahan, 1966). Further work, (McClenahan & Urtnowski, 1967) suggests that not only is the fall in compliance related to tidal volume but also to the frequency of ventilation when experiments are done at room temperature. When the lungs of rats and dogs were ventilated at 37°C these workers found no change in compliance.

Shephard (1962) found in anaesthetised rabbits during IPPV that the functional residual capacity increased when the rate of ventilation exceeded 56/minute. Compliance fell at rates exceeding 60/minute.

PATHOLOGICAL CONDITIONS ASSOCIATED WITH
ALTERED PULMONARY SURFACE ACTIVITY

Alteration of pulmonary surface activity is to be expected in conditions which damage the alveolar epithelial lining or which cause physical inactivation of pulmonary surfactant. Injury to alveolar cells by infection, ischaemia, or ionising radiation causes reduction of surface activity, (Clements & Tierney, 1965); whereas surface active lipids or enzymes, such as phospholipase, inactivate pulmonary surfactant.

There are numerous pathological and surgical conditions associated with reduced pulmonary surface activity; the following are the most important:-

1. Atelectasis.
2. Respiratory Distress Syndrome of the newborn.
3. Impaired pulmonary perfusion,
 - (a) pulmonary artery occlusion,
 - (b) extracorporeal circulation.
4. Fluid in the lungs,
 - (a) pulmonary oedema,
 - (b) amniotic fluid, and drowning.
5. Bilateral cervical vagotomy.
6. Lung transplantation.
7. Pneumonia.
8. Oxygen toxicity.
9. Carbon dioxide poisoning.
10. Inhalation of noxious gases.
11. Radiation pneumonitis.

ATELECTASIS

- Atelectasis or collapse may be extensive or focal.

Extensive atelectasis:

Pleural effusion, pneumothorax or bronchial obstruction can cause large segments of the lung to collapse. In these conditions altered pulmonary surface activity is the effect rather than the cause of the atelectasis; reinflation of an atelectic lung requires a pressure of at least 10 - 15 cms.H₂O, indicating reduced surface activity, (Lindskog & Bradshaw, 1934).

Reduction of pulmonary surface activity occurs twenty-four hours after the onset of atelectasis induced by pneumothorax, (Avery & Chernick, 1963). Forty-eight hours after bronchial ligation there is loss of pulmonary surfactant, (Tooley, Gardner, Thung & Finley, 1961). Return to normal surface activity occurs after fifty days, by which time adequate collateral bronchial circulation is established, (Finley, Swenson, Clements, Gardner, Wright & Severinghaus, 1960).

Sutnick & Soloff (1963) found reduced surface activity within minutes of 1. bronchial ligation, and 2. atelectasis produced by applying an external pressure to the pleural surface.

High surface tensions were found in extracted material from collapsed lungs; normal surface tensions were found when the lung was reinflated prior to extraction, (Yeh, Ellison, Manning, Hamilton & Ellison, 1965). There was no difference in the phospholipid content between the collapsed lungs and the controls; the period of collapse varied from 1 - 63 days.

The mechanism causing reduction of surface activity of extracts of collapsed lung, is not clear. It does not correlate with the pressure - volume relationships obtained after atelectasis of this type. Decreased compliance has been reported when chronically atelectic lungs are re-aerated, (Benfield, Rattenborg, Gago, Nigro & Adams, 1962; Carlson, Classen, Gollan, Gobbel,

Sherman & Christensen, 1958).

Focal atelectasis:

Focal atelectasis has been attributed to 1. local increase in surface tension, (Clements, Hustead, Johnson & Gribetz, 1961) 2. low lung volumes, (Von Neergaard, 1934) 3. obstruction of the terminal airways, (Craig, Fenton & Gitlin, 1958). 4. contraction of the smooth muscle fibres in the walls of alveolar ducts, (Halmagyi & Colebatch, 1961; Nadel, Colebatch & Olsen, 1962) and 5. shallow respiration associated with general anaesthesia or severe pain, (Burbank, Cutler & Sbar, 1961; Hamilton, 1961; Von Neergaard, 1934).

Focal atelectasis is most marked in areas of the lungs where inspiratory excursion is least; it is temporarily reversed by occasional deep inspirations, (Mead & Collier, 1959).

RESPIRATORY DISTRESS SYNDROME OF THE NEWBORN

Respiratory Distress Syndrome, (hyaline membrane disease, atelectasis neonatorum) is characterised by 'pulmonary atelectasis associated with congestion, the presence of eosinophilic hyaline membranes within dilated alveolar ducts, and intrapulmonary haemorrhage', (Hutchison, 1964). The hyaline membrane is mainly fibrin although it does not give the usual histochemical reactions of this substance, (Gitlin & Craig, 1956).

There are several theories concerning the aetiology of this condition; but none have been proved.

The role of pulmonary surfactant in its pathogenesis is uncertain; but is certainly implicated. Avery & Mead (1959) found that in infants who had died of this condition, there was evidence that pulmonary surfactant was absent; low compliance, high surface tensions of extracted lung material and absence of osmiophilic inclusion bodies in the type II alveolar cells. This has been subsequently confirmed, (Pattle, Claireaux, Davies & Cameron, 1963; Reynolds,

Orzalesi, Motoyama, Craig & Cook, 1965).

Deficient pulmonary surfactant could account for all the cardiopulmonary abnormalities of the condition, (Reynolds & Strang, 1966); for example, it could cause atelectasis and decreased lung compliance, (Karlberg, Cook, O'Brien, Cherry & Smith, 1954) together with uneven and, in severe cases insufficient, alveolar ventilation, (Blystad, 1956). The large right to left shunt which is typical of this condition, (Prod'hom, Levison, Cherry & Smith, 1965) could be explained by an increase in pulmonary vascular resistance caused by hypoxaemia, hypercapnia or lung collapse all of which can result from absence of pulmonary surfactant, (Cook, Drinker, Jacobson, Levison & Strang, 1963).

The extent to which lack of pulmonary surfactant is caused simply by biochemical immaturity of the lung remains unknown. Immature lambs delivered without asphyxia at a gestational age when pulmonary surfactant is undeveloped rapidly die from an illness very similar to respiratory distress syndrome of the newborn human infant, (Reynolds, Jacobson, Motoyama, Kikkawa, Craig, Orzalesi & Cook, 1965).

The incidence of respiratory distress syndrome in human infants is increased by conditions causing prenatal asphyxia, (Cohen, Weintraub & Lilienfeld, 1960); in monkeys asphyxia causes a similar condition, (Adamson, Behrman, Dawes, James & Koford, 1964).

During asphyxial episodes foetal lambs inspire amniotic fluid, (Howatt, Humphreys, Normand & Strang, 1965); but when large amounts of amniotic fluid were introduced into one lung of foetal lambs and the lungs ventilated separately, there was no difference in the surface properties of the two lungs.

IMPAIRED PULMONARY PERFUSION

Pulmonary artery ligation:

Experimental unilateral pulmonary artery occlusion causes a rapid fall in compliance and ventilation of the lung supplied by it, (Severinghaus, Swenson, Finley, Lategola & Williams, 1961; Swenson, Finley & Guzman, 1961). Several days later, there is pulmonary oedema, capillary congestion and focal atelectasis, (Schlaepfer, 1926; Karsner & Ash, 1912-13; Huber & Edmunds, 1967). These pathological changes are thought to result in part from increased alveolar surface forces (Finley, Tooley, Swenson, Gardner & Clements, 1964; Giammona, Mendelbaum, Foy & Bondurant, 1966).

Hypoperfusion of the pulmonary artery bed eventually leads to deficiency of pulmonary surfactant, (Chu, Clements, Cotton, Klaus, Sweet, Thomas & Tooley, 1965). The reduction of compliance is proportionately greater than the reduction of ventilation, (Severinghaus et al, 1961).

Several weeks after unilateral pulmonary occlusion the pathological changes are reversed, functional changes return to normal, (Chernick, Hodson & Greenfield, 1966; Finley, Swenson, Clements, Gardner, Wright & Severinghaus, 1960; Bloomer, Harrison, Lindskog & Liebow, 1949); this is due to gradual increase of collateral blood flow, (Weibel, 1960).

The surface tension of extracts of lungs increases within 15 hours following unilateral pulmonary artery occlusion, (Finley, Swenson et al, 1960; Finley, Tooley et al, 1964; Said, Harlan, Burke & Elliot, 1968; Chernick et al, 1966; Giammona et al, 1966; Tooley, et al, 1961); this is associated with decreased phospholipid content especially dipalmitoyl phosphatidyl choline in the extracts, (Morgan & Edmunds, 1967; Said et al, 1968).

Secondary infection following unilateral occlusion of the pulmonary artery has been reported as a possible cause of decreased surface activity, (Pattle

& Burgess, 1961); but this is disputed by others, (Tolley et al, 1961).

Pulmonary embolism induced by microscopic polyester particles causes a reduction of lung compliance, (Halmagyi & Colebatch, 1961; Nadel, Colebatch & Olsen, 1962); this is thought to be due to reflex spasm of alveolar duct smooth muscle.

Extracorporeal circulation:

Pulmonary congestion after circulatory by-pass procedures is well recognised as a post-operative complication, (Dodrill, 1958). The lungs, at post mortem examination show, vascular congestion, and focal atelectasis. They often have 'proteinaceous fluid in the alveoli, (Baer & Osborn, 1960). Histologically there are, hyaline membranes and swelling of alveolar cells, (Kolff, Effler & Graves, 1960). Human lungs have been examined electronmicroscopically following cardio-pulmonary by-pass, (Schulz, 1962) and show thickening of the alveolar wall, pericapillary oedema and swelling of the alveolar cells.

Excessive pulmonary vascular pressures during cardio-pulmonary by-pass in dogs leads to acute congestion and focal haemorrhage, (Muller, Littlefield & Damman, 1958); but prevention of left atrial overload does not alter the occurrence of focal atelectasis, (Kottmeier, Adamson, Stuckey, Newman & Dennis, 1958).

Baer & Osborn, (1960) suggested that denatured protein in extracorporeal circulating blood might contribute to the production of the pulmonary lesions. This has since been confirmed, (Lee, Krumbaar, Fonkalsrud, Schjeide & Maloney, 1961).

Release of free lipids into systemic blood has been found after cardio-pulmonary by-pass, (Owens, Adams, McElhannon & Youngblood, 1959). Free fatty acids (oleic acid and sodium oleate) have been injected intravenously into dogs, (Jefferson & Necheles, 1948); this resulted in pulmonary oedema and congestion,

and swelling of alveolar cells.

When normal dogs are transfused with blood which has been passed through a pump oxygenator for four hours, they develop acute pulmonary oedema and congestion, (Tooley, Finley & Gardner, 1961). When this blood is mixed with extracts of normal lungs, the minimum surface tension obtained on the surface tension balance is about 18 dynes/cm.; the control values are between 1 - 8 dynes/cm. This suggests that blood which has been passed through a pump oxygenator contains substances which block the surface activity of lung extracts. Tierney & Johnson, (1961) found that when cholesterol or oleic acid was added to the surface of lung extracts in the surface tension balance, the minimum surface tension obtained was 18 dynes/cm.

From these studies it seems probable that during cardio-pulmonary by-pass procedures, the following sequence of events occurs:-

1. Plasma protein is denatured,
2. this releases free lipids from lipoprotein molecules,
3. the free lipids may then cause pulmonary embolism, damage alveolar cells directly or interfere with pulmonary surfactant,
4. this results in focal atelectasis, pulmonary oedema and congestion.

These events occur more frequently when the lungs are underventilated and when pulmonary vascular pressure is allowed to rise unchecked, (Kottmeier et al, 1958).

FLUID IN THE LUNGS

Pulmonary oedema:

Pulmonary surfactant has been attributed as a possible factor in the prevention of pulmonary oedema, (Pattle, 1958; Clements, 1961). There is a balance of forces on the pulmonary capillaries tending to limit the movement of fluid out of the vascular compartment. Colloid osmotic pressure, tending to keep fluid within the capillaries, is almost balanced by capillary hydrostatic pressure, tissue fluid osmotic pressure and alveolar surface tension, tending to draw fluid out into the

pericapillary space. Clements (1962) has postulated that the presence of pulmonary surfactant on the alveolar surface lowers the 'porosity or permeability of the membrane'.

Pulmonary oedema, induced with intravenous dextran, is associated with increased alveolar surface forces and reduced surface activity of lung extracts, (Said, Avery, Davis, Davis, Banerjee & El-Gohary, 1965).

Taylor & Abrams (1964) found that fibrinogen, as a possible constituent of alveolar transudate, inactivated extracted pulmonary surfactant.

Amniotic fluid; and drowning

Pulmonary surface activity is reduced when amniotic or allantoic fluid is instilled down the airway and the lung ventilated, (Johnson, Permutt, Sipple & Salem, 1964; Johnson, Salem & Holzman, 1964).

Reduced surface activity has also been shown in guinea pigs after drowning, (Manktelow & Hunt, 1967); the most marked changes occurred after drowning in distilled water and the least after drowning in sea water.

BILATERAL CERVICAL VAGOTOMY

Bilateral cervical vagotomy in guinea pigs causes an increase in the minimum surface tension of extracted lung material, (Tooley, Gardner et al, 1961), and is associated with a reduction in the number of osmiophilic inclusion bodies in the type II alveolar cell, (Klaus, Reiss et al, 1962). Pulmonary oedema and the formation of hyaline membranes have been reported in rabbits after vagotomy, (Farber, 1937; Miller, Behrle & Gibson, 1951).

LUNG TRANSPLANTATION

Giammona, Waldhausen & Daly, (1964) found that in dogs which were autotransplanted or homotransplanted with denervated lungs, there was no alteration in pulmonary surface activity during the first twenty-four hours.

Thereafter the autotransplanted lung remained normal, whereas the homotransplanted lung showed reduced surface activity of extracted lung material and also reduced compliance of the whole lung.

On the other hand, it has been shown that homotransplantation does not alter pulmonary surface activity until twenty days post-operatively, (Yeh, Ellison & Ellison, 1964); this coincided with the onset of organ rejection.

PNEUMONIA

Bubbles expressed from pneumonic lungs are often unstable, (Pattle & Burgess, 1961). There is loss of normal surface activity of lung extracts from pneumonic lungs, (Sutnick & Soloff, 1964); this is thought to be due to proteolytic enzymes produced either by the invading organism or by the host. Influenzal pneumonia in mice causes rupture of alveolar cells, (Hers, Mulder, Masurel, Kuip & Tyrrell, 1962) and in humans who have died of Asian influenza, the lungs show atelectasis and hyaline membrane formation, (Martin, Dunin, Gottlieb, Barnes, Liu & Finland, 1959).

OXYGEN TOXICITY

Exposure of rats, (Loubiere & Pfister, 1966; Pfister, Fabre, Loubiere & Violette, 1964; Hemingway & Williams, 1950) and guinea pigs, (Ambrus, Pickren, Weintraub, Niswander, Ambrus, Rodbard & Levy, 1968) to pure oxygen causes atelectasis, pulmonary oedema, vascular congestion and hyaline membrane formation after two to three days.

Collier, (1963) found reduced surface activity of lung extracts of rats and rabbits which had died in pure oxygen. Other workers have failed to detect change in surface activity on exposure to pure oxygen even when pulmonary oedema was present, (Bondurant & Smith, 1962; Fujiwara, Adams & Seto, 1964; Pattle & Burgess, 1961).

CARBON DIOXIDE POISONING

Exposure to 15% CO₂ causes high surface tensions in extracted lung material and reduction in the number of osmiophilic inclusion bodies in the type II alveolar cell, (Schaefer, Avery & Bensch, 1964). These changes were most marked after twenty-four hours exposure; this coincided with the time when the respiratory acidosis was most severe. When the respiratory acidosis was compensated recovery of normal surface activity occurred.

INHALATION OF NOXIOUS GASES

Inhalation of mercury vapour causes erosive bronchitis, and bronchiolitis, intense vascular congestion and hyaline membrane formation. These are associated with a reduction of pulmonary surface activity, (Capers, 1961). Inhalation of phosgene, cadmium oxide, and black smoke did not alter the stability of lung bubbles in the early stages, (Pattle, 1967). Ozone increases the minimum surface tension of lung extracts and causes atelectasis in mice exposed to it, (Mendenhall & Stokinger, 1962).

Cigarette smoke has been shown to lower the surface tension of extracted animal and human lung material by as much as 33%, (Bondurant, 1960; Miller & Bondurant, 1962). Such a change occurring in vivo would alter alveolar stability, favouring the development of overdistension of alveoli.

RADIATION PNEUMONITIS

Radiation pneumonitis is often associated with focal atelectasis and hyaline membrane formation, (Warren & Gates, 1940), leading to reduced lung compliance.

PART I

PULMONARY SURFACE ACTIVITY STUDIES

'The lungs are emulsions of air in tissue and the bulk properties of such a finely dispersed system depend to a large extent on the properties of the interphase between them.'

(Clements, 1962)

PRESSURE-VOLUME STUDIES

INTRODUCTION

Analysis of the mechanical properties of excised lungs is usually accomplished by relating the static volume change to a corresponding change in elastic recoil force, measured as a change in airway pressure.

Lung compliance is the ratio of the change in volume to the change in recoil pressure, ($\Delta V/\Delta P$). It is usually measured in l/cm H₂O, in humans and large mammals, or in cc/cm H₂O, in small mammals. Because the relationship between compliance and recoil force is an inverse one, conceptual difficulties sometimes arise when relating one to the other. The term elastance which is the reciprocal of compliance (i.e. $\Delta P/\Delta V$) is frequently used to avoid this difficulty and is measured in cm H₂O/l or cm H₂O/cc.

Mechanical lung forces:

There are other forces besides elastic recoil which must be considered, when evaluating changes in airway pressure under dynamic conditions: like many distensible organs, such as blood vessels and gut, the pressure required to overcome inertia and the resistance to deformation, must be taken into account, (Radford, 1957).

Generally the resistance to deformation is related to the initial volume increment per unit time ($\delta V/\delta t$), while the forces of inertia depend on the acceleration of 1. air within the airways, and 2. lung tissue elements.

Rohrer (1925) first proposed the division of the mechanical properties of lung into three basic components; inertia, resistance and elasticity. He concluded that inertial forces in the lung are insignificant. This view has been confirmed, (Mead & Whittenberger, 1953; Nisell & Dubois, 1954). It has been shown that the major factor in total lung inertia is the inertia of air flow, (Mead, 1955).

Separation of resistive (or viscous) forces from elastic forces is a difficult procedure. This has been attempted under dynamic conditions, (Mead & Whittenberger, 1953; Nisell & Dubois, 1954; Von Neergaard & Wirz, 1927; Baylis & Robertson, 1939; Otis, Fenn & Rahn, 1950). In general the lung viscous forces vary with air flow rate in the airways; they are much less affected by the resistance to deformation of lung tissue, (Marshall & Dubois, 1956; McIlroy, Mead, Selverstone & Radford, 1955). Analysis of the mechanical properties of lungs under static conditions will therefore, afford a better estimate of elastic recoil forces.

Elastic properties and hysteresis:

Von Neergaard (1929) attempted the first static measurement of lung elastic forces. He found that surface forces accounted for the major part of total recoil forces. He completely emptied excised lungs, filled them with either a 7% solution of gum arabic, or air, and compared pressure-volume curves. The results of his experiments are summarised below.

Lungs filled with gum arabic	Lungs filled with air
Hysteresis negligible.	Hysteresis marked.
Opening pressure less than 1 cmH ₂ O.	Opening pressure 8 cmH ₂ O
Filling rapid and even.	Filling slow and patchy.
Maximally inflated at 10cmH ₂ O	Maximally inflated at 30cmH ₂ O
Deflation smooth and slightly sigmoid shaped curve.	Deflation uniform and markedly sigmoid shaped curve.

Hysteresis is the difference in the pressure-volume characteristics according to the direction of change in pressure. The opening pressure, which is the pressure which must be applied to the lungs before any appreciable change in volume occurs, was about 3 cmH₂O on initial liquid filling. The curve showed moderate hysteresis. This is thought to be due to the presence of bronchial mucus in the airways, (Radford, 1957). Subsequent liquid filling abolished the opening pressure effect and the hysteresis. On filling the lungs with air, the opening pressure effect and hysteresis are marked and are thought to be due to surface tension forces, (Mead, Whittenberger & Radford, 1957).

Numerical index of lung stability:

An index of the stability of the lung can be obtained from the deflation part of the pressure-volume curve. Gruenwald (1963) found good reproducibility when that part of the deflation curve between 10 and zero pressure was taken, since the relationship is essentially linear over this pressure range. The index he devised for human lungs is given by:

$$L = \frac{2V_5 + V_{10} - 3V_0}{2V_{\max} - 2V_0} \quad (\text{Equ. 1})$$

where V_5 , V_{10} and V_0 are the volumes at pressures of 5, 10 and zero cmsH₂O respectively. V_{\max} is the volume of the lungs when maximally inflated.

Gruenwald suggested that for small mammals the term V_0 should be omitted; since the pressure-volume relationships of many small mammals is characterised by relatively large lung volumes at zero pressure. The stability index is therefore, given by:

$$L = \frac{2V_5 + V_{10}}{2V_{\max}} \quad (\text{Equ. 2})$$

Normal values for equation 1, range from 0.8 to 1.4 and for equation 2, from 0.9 to 1.2.

Present study:

The present study sets out to establish normal pressure-volume relationships for the lungs of guinea pigs during air filling, and investigates the effect of hyperventilation on them. Lung compliance and stability indexes are compared for the pre and post-hyperventilation periods.

The lungs of seven living guinea pigs were removed and studied for pressure-volume characteristics before and after hyperventilation. The lungs of two animals were ventilated for 5 minutes, two for 10 minutes, two for 15 minutes and one for 30 minutes.

METHODS

Calibration of apparatus for pressure measurement:

Pressure was measured by a Greer electromanometer (type M3, Mercury Electronics, Scotland.) capable of measuring a differential pressure of up to 60 cmsH₂O (Plate XIII). The instrument was tested for linearity of response and calibrated at three monthly intervals.

The electromanometer was connected to the side arm of a water manometer having a range of 0-60 cmsH₂O. Stepwise increments of 1 cmH₂O were applied to the other arm of the water manometer using a sphygmomanometer hand pump, up to a total differential pressure of 60 cmsH₂O. The response of the electromanometer was simultaneously recorded on a pen recorder (YEW x.x-y Recorder, Yokogawa Electric Works, Japan.).

The response was found to be linear up to a differential pressure of 45 cmsH₂O. Above this it became non-linear with an error of about +1% at 50 cmsH₂O, and +5% at 60 cmsH₂O. The maximum pressure never exceeded 45 cmsH₂O in any of the experiments performed.

Pre-experimental calibration check:

The electromanometer was checked at the start of every experimental day and calibrated. It was set up as described above. Differential pressures of 10, 20 and 30 cmH_2O were applied to the water manometer. The recorded pressure readings were compared with actual pressures and any necessary adjustments to the electromanometer were made.

Method of performing pressure-volume manoeuvres:

The main stem of a 'Y' tube was tied into the trachea. One side arm was connected to a Greer electromanometer, while the other was connected to a calibrated syringe. Pressure was recorded on a pen recorder. Successive slow injections of 1 cc of air were made, allowing 1 minute between each step for equilibration of the static recoil pressure within the lungs. The maximum inflation volume, henceforth termed the total volume of the lungs, was taken as having been reached when the pressure started to rise steeply for a very small volume increment. Deflation was effected by an exactly reversed procedure.

Pressure volume curves were obtained by plotting volume against the static recoil pressure, for each step during inflation and deflation respectively.

Experimental procedure:

Seven guinea pigs (G.U. Physiol./Biochem. strain) were anaesthetized with intraperitoneal pentobarbitone sodium (dose 50mg/kg). A tracheostomy was performed and the trachea cannulated with a 'Y' tube. The side arms of the 'Y' tube were clamped and a sternum splitting incision was quickly made. The anterior chest wall was widely separated and the trachea, lungs, heart and great vessels were dissected out en bloc. The lungs were then allowed to collapse slowly by slackening one of the clamps on the 'Y' tube. The heart and great vessels were carefully dissected away. The 'Y' tube was fixed to a clamp stand

with the lungs suspended vertically. The pleural surface of the lungs was periodically moistened with normal saline throughout the entire experiment.

Stepwise filling and emptying of the lungs, as described above, was carried out three times, so that a mean control pressure-volume curve could be drawn.

Ventilation of the lungs:

The lungs were attached to a Palmer small animal pump and ventilated. The tidal volume used was $3/4$ of the total volume of the lungs. The frequency was constant at 32/min. The lungs of two animals were ventilated for 5 minutes, two for 10 minutes, two for 15 minutes and one for 30 minutes. At the end of the ventilation period, three pressure-volume manoeuvres were performed on each of the lungs. Thus a mean post-ventilation pressure-volume curve could be drawn.

On completion of the experiment all lungs were maximally inflated under saline to check for air leaks. Measurements from lungs which showed evidence of air leakage were discarded. This resulted in only five animals being included in the study. The lungs of the two animals not included, had lacerations on the pleural surface caused by abrasion with exposed ribs. The operative technique was slightly modified to prevent this occurrence in subsequent experiments.

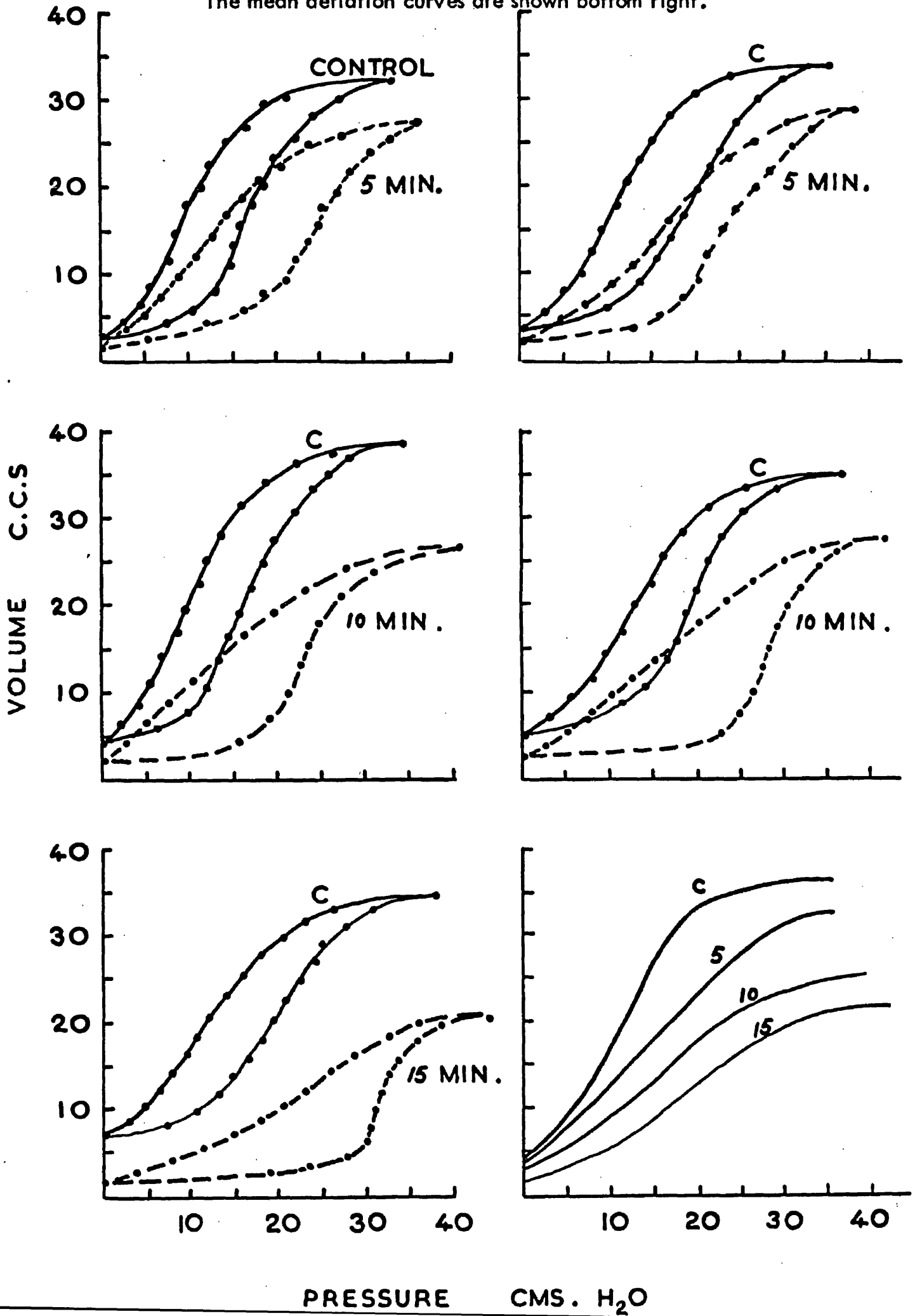
RESULTS

Pressure-volume curves:

The mean pre and post-hyperventilation pressure-volume curves are shown in fig. III , for each of the animals studied.

There was no significant difference between animals during the control period. Hysteresis between inflation and deflation curves was of similar magnitude in all cases. The opening pressure ranged from 6-8 cmsH₂O. The pressure

Fig. 111 - PRESSURE-VOLUME CURVES FOR EACH CORNER FIG.
The mean deflation curves are shown bottom right.



required to maximally inflate the lungs ranged from 25-30 cmH_2O , and the total volume of lungs ranged from 32-36 cc.

After hyperventilation the curves had rotated down and to the right. There was a tendency for hysteresis to increase progressively with the duration of hyperventilation. The deflation curve became progressively less sigmoid. The opening pressure and the pressure required to maximally inflate the lungs increased with hyperventilation, whereas the total volume of lungs fell progressively.

	Opening pressure	Pressure at total vol. lungs	Total vol. lungs
Control	7 cmH_2O	27.5 cmH_2O	34 cc
5 mins	11.5 "	36.5 "	28.5 cc
10 mins	15.5 "	42 "	26 cc
15 mins	24 "	44 "	23 cc

The values shown above are means. The standard deviations were insignificantly small.

Lung compliance was calculated from the deflation curves by computing, for each animal the mean ratio of volume to pressure. Six sets of values were extracted from each curve at points corresponding to 5, 10, 15, 20, 25 and 30 cmH_2O pressure. The results are shown in table 1. The mean compliance for all animals during the control period was 1.7 $\text{cc}/\text{cmH}_2\text{O}$. Hyperventilation caused compliance to fall so that after 15 min. it was only 30% of the initial value.

The difference between all four ventilation groups were highly significant. Student *t* values and probabilities are shown.

TABLE I
LUNG COMPLIANCE RESULTS IN cc/cm H₂O

	CONTROL	5MIN	10MIN	15MIN
	1.3	1.3	0.5	0.2
	1.2	1.6	0.5	0.2
	1.5	1.5	0.7	0.4
	1.6	1.4	0.8	0.5
	1.9	1.4	0.9	0.5
	2.0	1.4	1.0	0.6
	2.0	1.4	1.0	0.6
	2.0	1.3	1.0	0.6
	2.0	1.3	1.0	0.7
	2.0	1.3	1.0	0.7
	1.9			0.6
	1.6			0.6
	1.5			
	1.4			
MEAN	1.71	1.39	0.84	0.52
S.D.	±0.29	±0.10	±0.21	±0.17
S.E.M.	±0.079	±0.030	±0.065	±0.048
	$t_{22} = 3.27$ P < 0.01	$t_{18} = 7.78$ P < 0.001	$t_{20} = 4.11$ P < 0.001	

Lung stability indexes were computed for each of the lungs studied using equation 2 on page 38. The values are shown below.

	Control	5 min	10 min	15 min
Mean	0.57	0.49	0.31	0.10
S.D.	±0.04	±0.03	±0.03	±0.04
S.E.M.	±0.01	±0.01	±0.01	±0.02
	$t_{19} = 5.67$ $P < 0.001$	$t_{10} = 18.0$ $P < 0.001$	$t_7 = 12.39$ $P < 0.001$	

The differences between the four ventilation groups were highly significant in all cases. Hyperventilation caused the stability index to fall progressively. After 15 min. it had decreased to 17% of the control value.

DISCUSSION

It is evident from the pressure-volume relationships for the control lungs that guinea pigs are similar in this respect to other mammals. The values for compliance are similar to those previously reported for guinea pig lungs, (Agostoni, Thimm & Fenn, 1959; Hild & Bruckner, 1956). There are no previous studies reported which have measured the stability index of guinea pig lungs. The stability index of rat lungs lies within the range 0.38-0.96; for rabbit lungs it lies within the range 0.92-1.21, (Gruenwald, 1963). The guinea pig stability index in the present study was 0.57. Compliance of rat lungs however, has been found to be consistently higher than guinea pig lungs, (Agostoni et al, 1959). This might suggest that the stability index is a less accurate estimate of pulmonary surface activity than lung compliance.

Hyperventilation reduced compliance and the stability index, and decreased

the total volume of lungs. It increased the opening pressure and the pressure required to maximally inflate the lungs. This would indicate that the factors which maintain the normal pressure-volume relationship were progressively altered, and as a consequence more mechanical work was being performed. The forces maintaining the normal pressure-volume relationship are generally accepted as being of two types, tissue elastic forces and surface forces.

Since it has been shown that hyperventilation does not alter tissue forces, (McClenahan & Urtnowski, 1967), one can conclude that the predominant effect is a steady degradation of normal surface activity. This would lead to maintained high surface tensions within the lung with a tendency to premature collapse.

BUBBLE STABILITY METHOD

INTRODUCTION

The stability ratio of bubbles expressed from the lung, has been used as an index of pulmonary surface activity, (Pattle, 1958; Howatt & Strang, 1965; Edmunds & Huber, 1967; Sekulic, Hamlin, Ellison & Ellison, 1968; Slavkovic, Wingo, Ellison & Ellison, 1968).

The stability ratio of lung bubbles is the ratio of the final surface area to the initial surface area. It is calculated by dividing the sum of the square of the diameters of a group of bubbles, 20 minutes after they have been expressed from the lungs, by the sum of the squares of their initial diameters.

METHOD

Lung bubble stability ratios were calculated for the lungs of three control guinea pigs and compared with those obtained for three guinea pigs following hyperventilation for 10 minutes.

Procedure:

Six guinea pigs were anaesthetized with intraperitoneal pentobarbitone sodium (dose 60mg/kg). A wide thoracotomy was performed and the lungs removed intact. After experimenting with several techniques of preparing lung bubbles, the following method was finally adopted.

Lung samples, which in most cases were complete lobes, were taken from the upper and from the lower lobes of both lungs of each animal. The four pieces of lung were inflated with air from a syringe fitted with a number one needle. A small piece of lung tissue was grasped with a pair of forceps. Lung bubbles were squeezed into a drop of distilled aerated water, on a glass cover

slip. A hanging drop preparation was made by inverting the cover slip onto a glass slide with a hollow ground depression. This was immediately viewed under a light microscope; a suitable field was chosen, containing about twenty bubbles.

The light source was an infra-stage tungsten lamp which was only switched on during the counting procedures. Exactly one minute after the hanging drop preparation had been made, the diameters of the bubbles were measured using an integrated eyepiece micrometer (Leitz, type K, magnification $\times 8$). Measurement of the diameters was repeated after exactly 20 minutes.

Continuous viewing of the bubbles between counting procedures was achieved using a tungsten lamp placed about two feet away from the microscope stage, and angled to it. This prevented any thermal effect on the bubbles. Continuous viewing was performed to ensure that the same group of bubbles was used for measurement of diameters, initially and after twenty minutes.

Three of the animals used in this study were used as controls in which the lungs were removed immediately after thoracotomy. Three were ventilated for 10 minutes, with a Palmer small animal pump, at a tidal volume of $3/4$ of the total volume of the lungs and at a rate of 32/min. Measurement of the total volume of the lungs was by the method described on page 40. Samples of lung tissue were obtained at the end of the ventilation period by the method described above for the control lungs.

Eight hanging drop preparations were obtained for each animal. The groups of bubbles used for bubbles stability measurement had, in all cases, diameters within the range $30 - 70\mu$.

RESULTS

Sampling areas:

There was no significant difference between the four lung areas used for

sampling, in either control or ventilated lungs, (Table II). In the control group the difference between the upper right and the upper left lobes was just significant at $P = 0.05$; but there was no difference between the same areas in the ventilated group. This difference is predominantly due to data from one animal for these areas (animal 3, Table III).

Control and hyperventilated lungs:

The difference in overall mean stability ratios between the control and the ventilated groups, was highly significant, at $P < 0.001$ (Table IV). In all cases the individual sampling areas were significantly different between control and ventilated groups (Table II).

Hyperventilation reduced the stability ratio of lung bubbles from each animal and from each area sampled, by about 11%.

DISCUSSION

Normal stability ratios for most mammals range from 0.8 - 1 (Pattle, 1965). The mean value found in this study for the control guinea pigs was 0.84.

The difference between stability ratios for the upper right and the upper left lobes, in the control animals, was of relatively low statistical significance, also the difference was predominantly due to data from animal number 3; therefore no difference between lobes can be affirmed from this study.

Hyperventilation reduced the mean stability ratio to 0.77. This means that after hyperventilation, lung bubbles are less stable and tend to reduce in size more rapidly. This implies that the surface tension of these bubbles is high. One might conclude that there has been an alteration to the surface forces in the lung as a result of hyperventilation which is reflected in the poor stability of bubbles expressed from them.

STABILITY RATIOS

AREA SAMPLED	MEAN CONTROL $\pm 1SD$	MEAN HYPERVENTILATED $\pm 1SD$	STUDENT t-TEST AND PROBABILITIES
UPPER RIGHT	0.817 \pm 0.06	0.773 \pm 0.07	$t_{10} = 3.12, p < 0.01$
LOWER RIGHT	0.837 \pm 0.06	0.787 \pm 0.06	$t_{10} = 3.55, p < 0.001$
UPPER LEFT	0.857 \pm 0.06	0.77 \pm 0.08	$t_{10} = 5.03, p < 0.001$
LOWER LEFT	0.853 \pm 0.06	0.77 \pm 0.07	$t_{10} = 5.89, p < 0.001$

Stability ratios for each area sampled and for the control and hyperventilated lungs

TABLE II

STABILITY RATIOS FOR CONTROL AND VENTILATED GROUPS

ANIMAL	UPPER RIGHT \pm 1 SD	LOWER RIGHT \pm 1 SD	UPPER LEFT \pm 1 SD	LOWER LEFT \pm 1 SD
1	0.81 \pm 0.06	0.84 \pm 0.06	0.84 \pm 0.06	0.87 \pm 0.06
2	0.83 \pm 0.06	0.83 \pm 0.06	0.87 \pm 0.06	0.85 \pm 0.07
3	0.81 \pm 0.06	0.84 \pm 0.06	0.86 \pm 0.05	0.84 \pm 0.05
4	0.75 \pm 0.06	0.79 \pm 0.06	0.77 \pm 0.07	0.77 \pm 0.07
5	0.80 \pm 0.07	0.79 \pm 0.05	0.77 \pm 0.07	0.77 \pm 0.06
6	0.77 \pm 0.08	0.78 \pm 0.08	0.76 \pm 0.09	0.77 \pm 0.09

Control

50

Hyper-
ventilated

Mean stability ratios for each animal and for each area sampled

TABLE III

ANIMAL	MEAN SR \pm SD	
1	0.84 \pm 0.06	CONTROLS
2	0.84 \pm 0.06	
3	0.84 \pm 0.06	
4	0.77 \pm 0.07	HYPERVENTILATED
5	0.78 \pm 0.07	
6	0.77 \pm 0.08	

 $t_{10} = 4.68, P < 0.001$

Mean control and hyperventilated group stability ratios

TABLE IV

AUTOFLUORESCENCE AND FLUOROCROME STAINING

INTRODUCTION

Autofluorescence is the property of spontaneous fluorescence when excited by ultra-violet light. Tissues which do not exhibit marked autofluorescence can be made to fluoresce by staining them with specific fluorochrome dyes. Lung tissue has been shown to fluoresce spontaneously, (Boland & Klaus, 1964; Hackney, Collier & Rounds, 1965; Hackney, Rounds & Schoen, 1963).

Organic molecules which autofluoresce are generally proteins or protein complexes, (Pearse, 1968); or lipids, (Udenfriend, 1962).

PRELIMINARY STUDIES

Several methods were investigated, for the preparation of thin sections of lung, suitable for fluorescent microscopy. Eleven rats and six guinea pigs were anaesthetized with intraperitoneal pentobarbitone sodium (dose 40 mg/kg and 60 mg/kg respectively): the following procedures were then carried out.

Perfusion fixation with formol-calcium:

A wide abdominal incision was made on two guinea pigs; the inferior vena cava (IVC) and the abdominal aorta were each cannulated. The animals were perfused via the IVC cannula, with normal saline, at 35° C, and at a perfusion pressure of 5 mmHg. The aortic effluent was collected in a glass flask. When the effluent became clear, indicating that the animal had been washed through with saline, the perfusate was switched to a solution of formol-calcium (conc. formalin, 10%; 10% aqueous CaCl₂ in H₂O, 10%; distilled H₂O, 80%) at the same temperature and perfusion pressure. Perfusion was continued for about 20 minutes.

A wide thoracotomy was performed and the heart and lungs were removed

en bloc. They were immersed in formol-calcium and stored overnight.

Blocks of fixed lung tissue, approximately 0.5 x 0.5 cms, were cut; then frozen in a stream of expanding CO₂. 5 μ thick sections were cut on a cold microtome.

Instillation of formol-calcium down the airway:

A wide thoracotomy was performed on four rats. The trachea, lungs and heart were removed en bloc. The lungs were degassed in a vacuum jar. A solution of formol-calcium was slowly injected down the trachea from a syringe. When the lungs were completely filled with formol-calcium, the trachea was tied and the lungs immersed in a bath of formol-calcium overnight.

Thin sections were cut as described above.

Rapid freezing in liquid nitrogen:

The lungs of one rat and one guinea pig were removed intact, following thoracotomy. The trachea was cannulated and the lungs were inflated with air from a syringe. They were then immersed in liquid N₂ at -170°C. The lungs had frozen solid within 10 minutes. Small pieces of lung were chipped away using bone forceps, and placed on a frozen chuck at -12°C. Thin sections were cut on a cryostat, placed on a cold glass microscope slide and allowed to thaw: the sections were then air dried.

The lungs of a further two rats were frozen in isopentane cooled to -170°C by liquid N₂. Sections were prepared by the same method described for freezing in liquid N₂.

Rapid freezing with solid CO₂:

Thoracotomy was performed on four rats and three guinea pigs. The lungs were removed intact. The lower right lobe was isolated and the right lower lobar bronchus cannulated. The lobe was filled with air from a syringe.

The lobe was then placed on a chuck cooled to -40°C with solid CO_2 ('Drikold', ICI). When the lobe was completely frozen, cryostat sections were cut, 5μ thick. These were placed on glass microscope slides and allowed to thaw. Some sections were air dried while others were hydrated with distilled water, during thawing.

All microscope slides used in this study were treated to ensure that they were perfectly clean, since dust will fluoresce under certain conditions. The sections obtained by the four different methods described above, were examined with a Zeiss, ultra-violet microscope (Ultraphot 11), fitted with a high pressure mercury lamp (HBO 200). A BG12/4mm exciter filter and interposed barrier filters were used.

Sections from all animals studied were stained for conventional microscopy with haemalum and eosin, the Periodic acid-Schiff method, Gordon and Sweets' method for reticulin, Bakers' acid haematin method for phosphatides, Landings' phosphomolybdic acid method for choline containing lipids, and also with the fluorochrome dyes, Phosphine 3R, and Rhodamine B. Details of these staining methods are given in appendix 1.

RESULTS

Autofluorescence:

Autofluorescence was only seen in the sections of lungs, prepared by rapid freezing with solid CO_2 .

Thin bright yellow-green fluorescent lines (peak wave length, 550nm) were seen lining the alveoli, on air dried sections. These fluorescent lines contrasted with the less intense, and slightly greener fluorescence (peak wave length 500nm) of the surrounding tissue, (Plate II). When a cover slip was placed over the sections and a drop of distilled water allowed to wet them, bubbles formed on

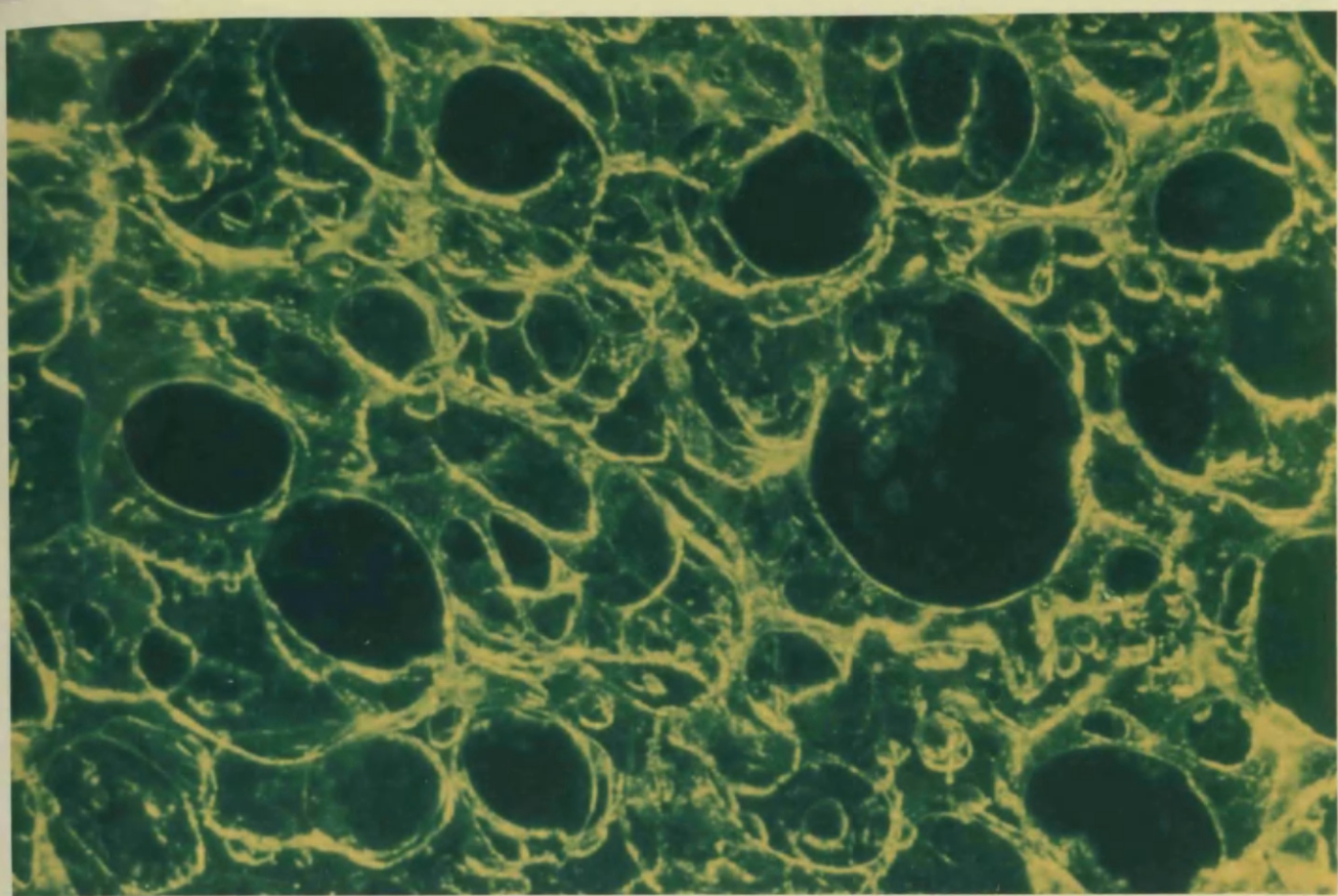


PLATE II

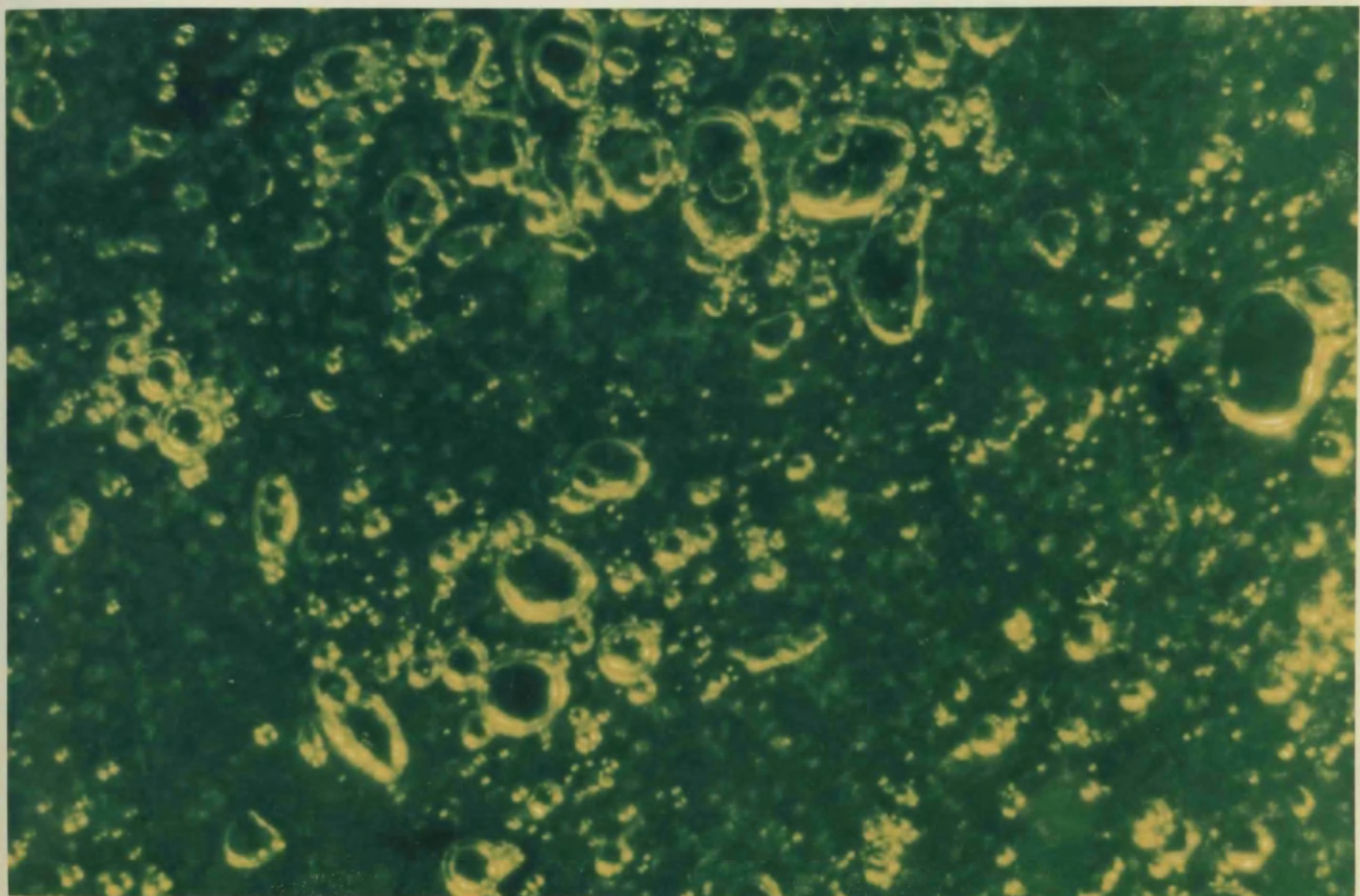


PLATE III

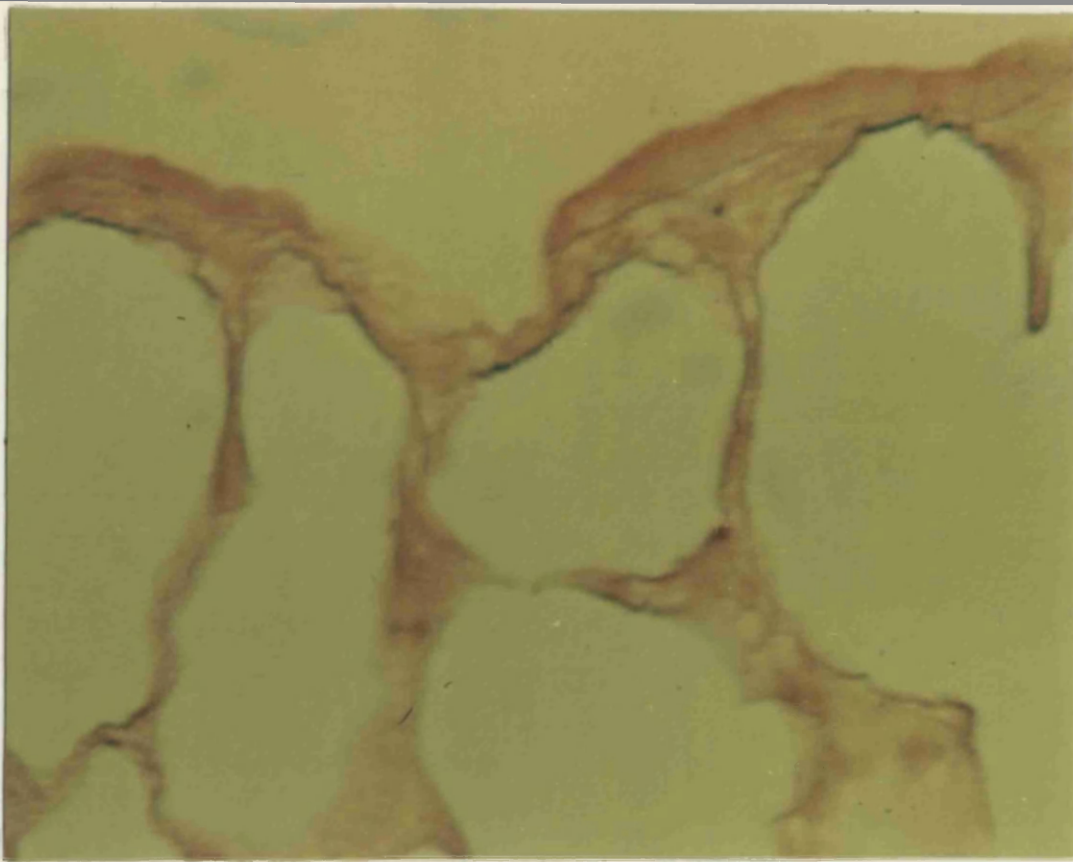


PLATE IV

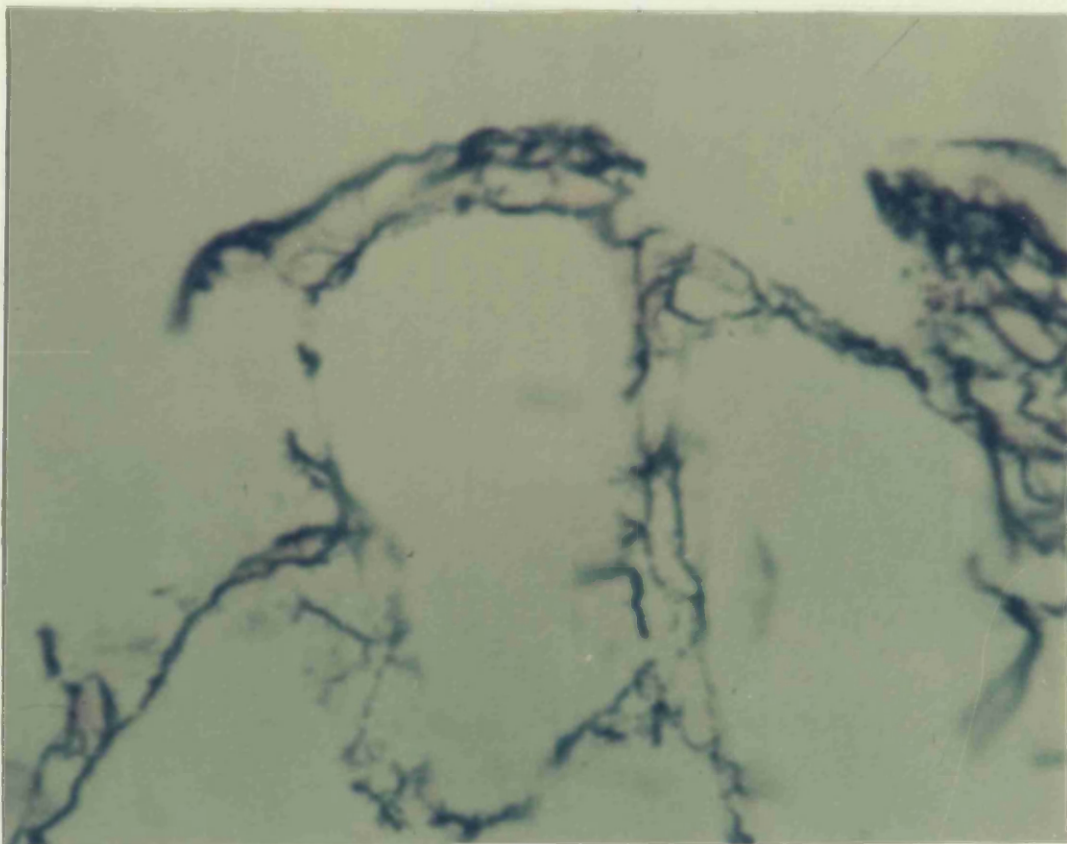


PLATE V

the surface of the sections. These bubbles showed bright fluorescence of the same intensity and peak wave length as the thin fluorescent lines, seen at the alveolar surface of air dried sections. (Plate III)

Fluorochrome staining:

Only sections from lungs prepared by freezing with solid CO₂ stained positively with the fluorochrome dyes. Sections stained with Phosphine 3R showed pale yellow fluorescence (peak wave length, 575nm) lining the alveoli. No background fluorescence was seen. Sections stained with Rhodamine B showed bright yellow fluorescence (peak wave length, 580nm) lining the alveolar surface; less intense yellow background fluorescence was seen.

In both cases RBC outer membranes also stained positively with these fluorochromes; the intensity of fluorescence was less than that seen at the alveolar surface.

Staining for conventional microscopy:

Acid haematin stained the alveolar surface; a thin blue black line was easily seen. Its distribution matched that of the fluorochrome dyes, but tended to be patchy.

Sections which had been stained with phosphomolybdic acid showed a thin dark blue layer at the alveolar surface.

Staining with the PAS method resulted in a thin deep purple line on the alveolar surface; contrasting with the pale red of the surrounding tissue (Plate IV). To exclude the possibility that this positive PAS reaction might be due to reticular fibres in the alveolar septa, sections were stained specifically for reticulin by the Gordon Sweet method. Numerous reticular fibres were seen around the alveoli, as thin wavy black lines. Their distribution did not correspond to the positive alveolar PAS reaction described above (Plate V).

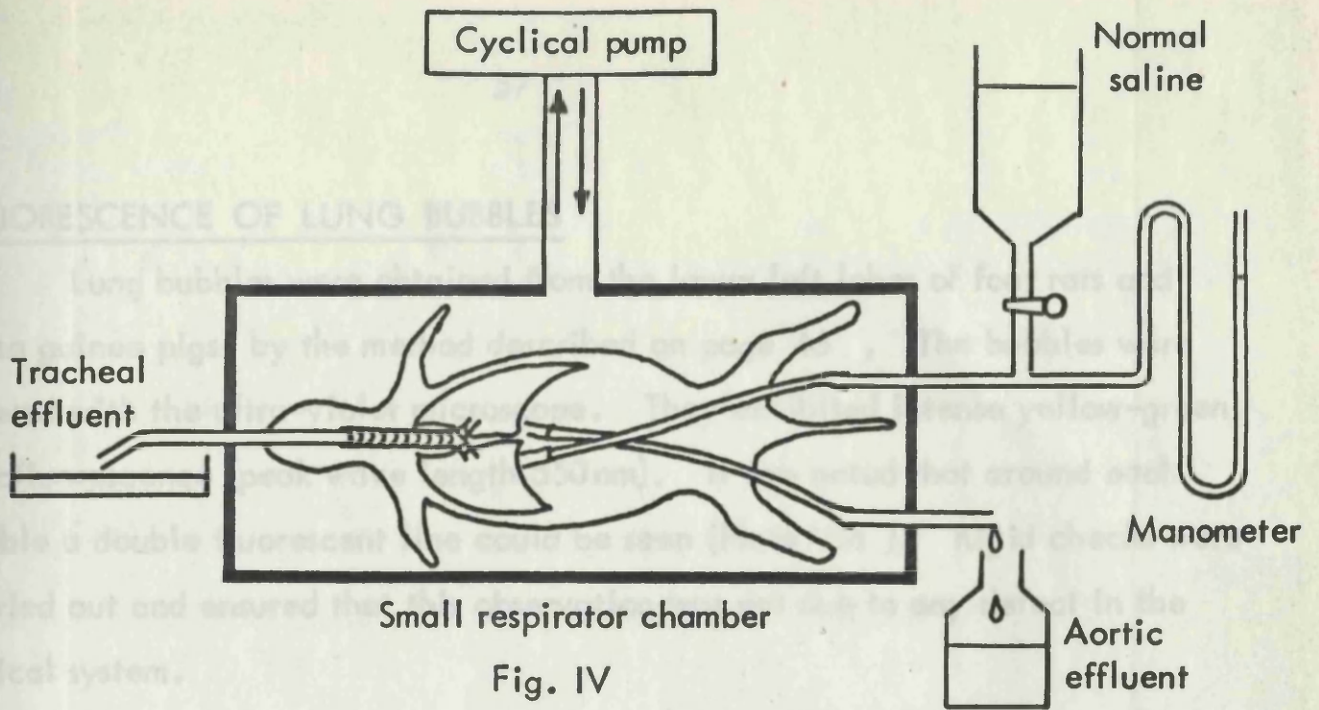


Fig. IV

FLUORESCENCE OF TRACHEAL FROTH

Two rats were anaesthetized with intraperitoneal pentobarbitone sodium (dose 40 mg/kg), and a wide thoracotomy was performed. The inferior vena cava, aorta and the trachea were each cannulated. The animals were perfused via the caval cannula with normal saline at 35°C, and at a perfusion pressure of 5 mmHg. The animals were placed in a small respirator chamber (Fig. IV), and ventilated with cyclical negative and positive chamber pressure. The effluent from the trachea was collected in a petri dish. A drop of the effluent was placed on a glass microscope slide and covered with a cover slip. These were then examined with the ultra-violet microscope.

RESULTS

Thin micelles of bright yellow-green fluorescent material were seen. These did not have any spherical configuration but were mostly long strands. The peak wave-length of these fluorescent strands was 550 nm. It was noted that the intensity of the fluorescence diminished rapidly under continuous ultra-violet excitation, unlike the autofluorescence seen on the air dried sections.

FLUORESCENCE OF LUNG BUBBLES

Lung bubbles were obtained from the lower left lobes of four rats and three guinea pigs, by the method described on page 46 . The bubbles were viewed with the ultra-violet microscope. They exhibited intense yellow-green autofluorescence (peak wave length 550nm). It was noted that around each bubble a double fluorescent line could be seen (Plate VIa). Rigid checks were carried out and ensured that this observation was not due to any defect in the optical system.

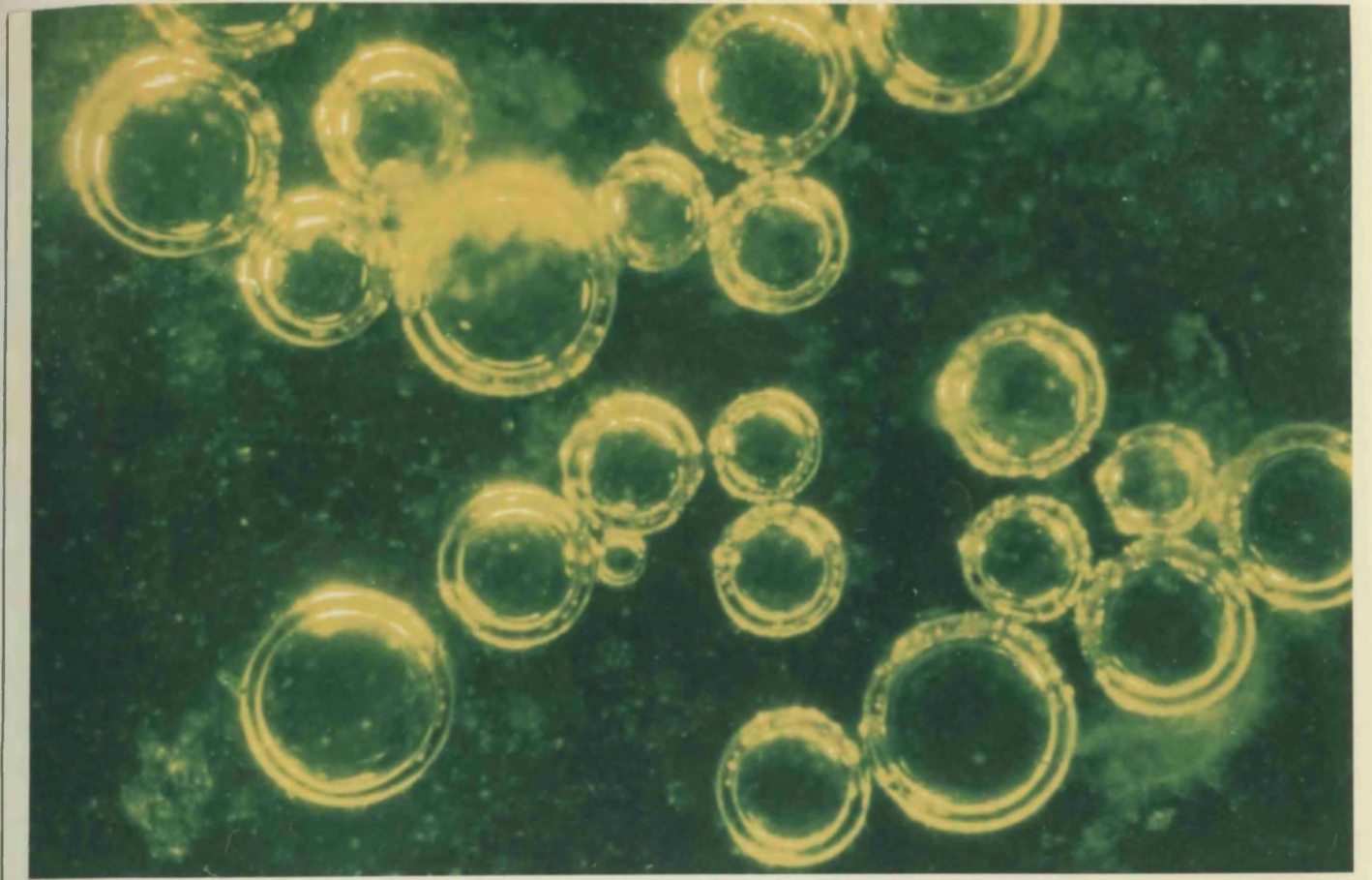
When bubbles were expressed from the lung onto a flat slide, they tended to adhere to one another. The double fluorescent lines were again present. As the bubbles dried, they assumed a polyhedral shape and the double fluorescent lines were seen to merge forming a single thicker line (Plate VIb). When the bubbles were completely dried, thin single fluorescent lines remained. They were now irregularly shaped and less intense; but had the same peak wave length as the fresh bubbles (Plate VIc).

EFFECT OF HYPERVENTILATION

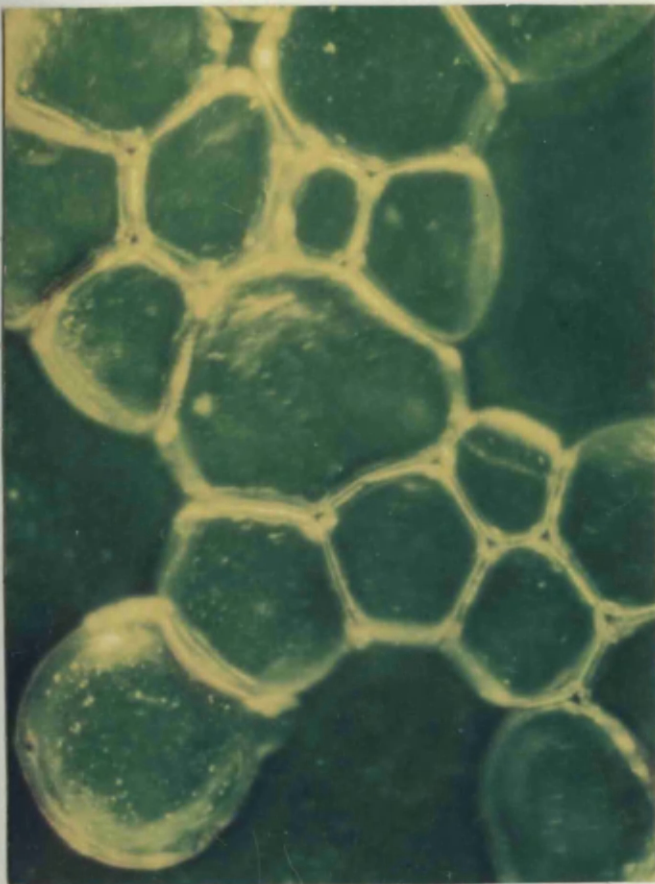
Procedure:

Twelve rats and six guinea pigs were anaesthetized with intraperitoneal pentobarbitone sodium (dose 40mg/kg and 60mg/kg respectively). The sternum was split and the anterior chest widely separated. The total volume of the lungs of all animals was measured prior to artificial ventilation, by the method described on page 40 . The animals were ventilated for 10 minutes with a Palmer small animal pump, at a tidal volume of $\frac{3}{4}$ of the total volume of the lungs. The ventilation rate for the rats was 60/min and for the guinea pigs was 32/min.

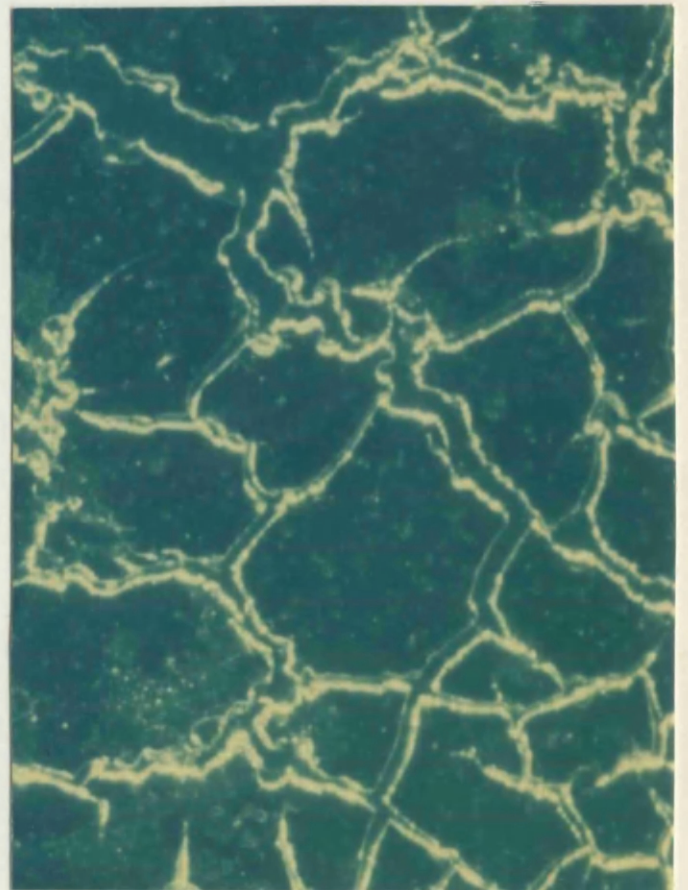
At the end of the period of hyperventilation, both lower lobes were removed. The lower right lobe was used for the preparation of 5 μ thick sections, by the method described for lungs which had been frozen with solid CO₂ (page 53)



A



B



C

The sections were examined with the ultra-violet microscope, both when hydrated and when air dried. The lower left lobe was used for the preparation of lung bubbles. Hanging drop preparations were made and examined with the ultra-violet microscope.

Results:

Autofluorescence of the alveolar surface was seen in all lungs which had been hyperventilated (Plate VII). It had a peak wave length of 550 nm. The intensity of the fluorescence varied considerably, from very weak to slightly less than that seen on sections of lungs prepared by the same method but not hyperventilated (see Plate II). Sections which were hydrated produced fluorescent bubbles on their surface. In all cases the intensity of fluorescence was less and the colour greener (peak wave length 500 nm) than that seen on hydrated sections of lungs which had not been hyperventilated. (Plate VIII)

Bubbles expressed from the hyperventilated lungs were in all cases brightly fluorescent (peak wave length 580 nm). No difference in the appearance of the bubble fluorescence could be affirmed between bubbles expressed from hyperventilated lungs and those from lungs which had not been hyperventilated: but qualitatively the bubbles from lungs which had been hyperventilated generally had less intense fluorescence.

DISCUSSION

Fixation with formol-calcium, and rapid freezing with liquid N₂ or with isopentane cooled by liquid N₂ altered the fluorescent and specific phospholipid staining properties of the alveolar wall. This was evidenced by the absence of autofluorescence, and lack of fluorochrome and lipochrome staining. Only those lungs which had been frozen with solid CO₂ showed autofluorescence and were

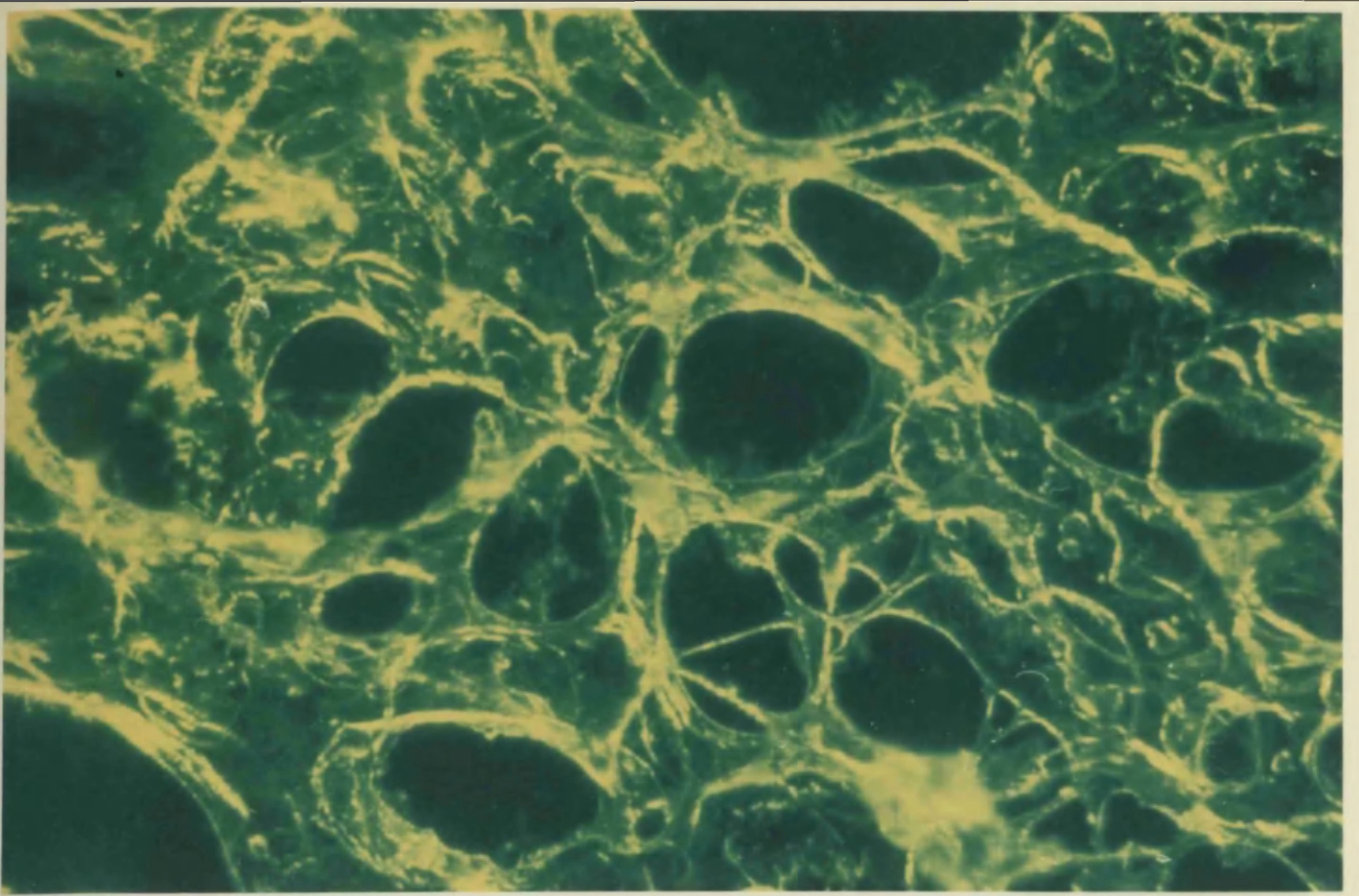


PLATE VII

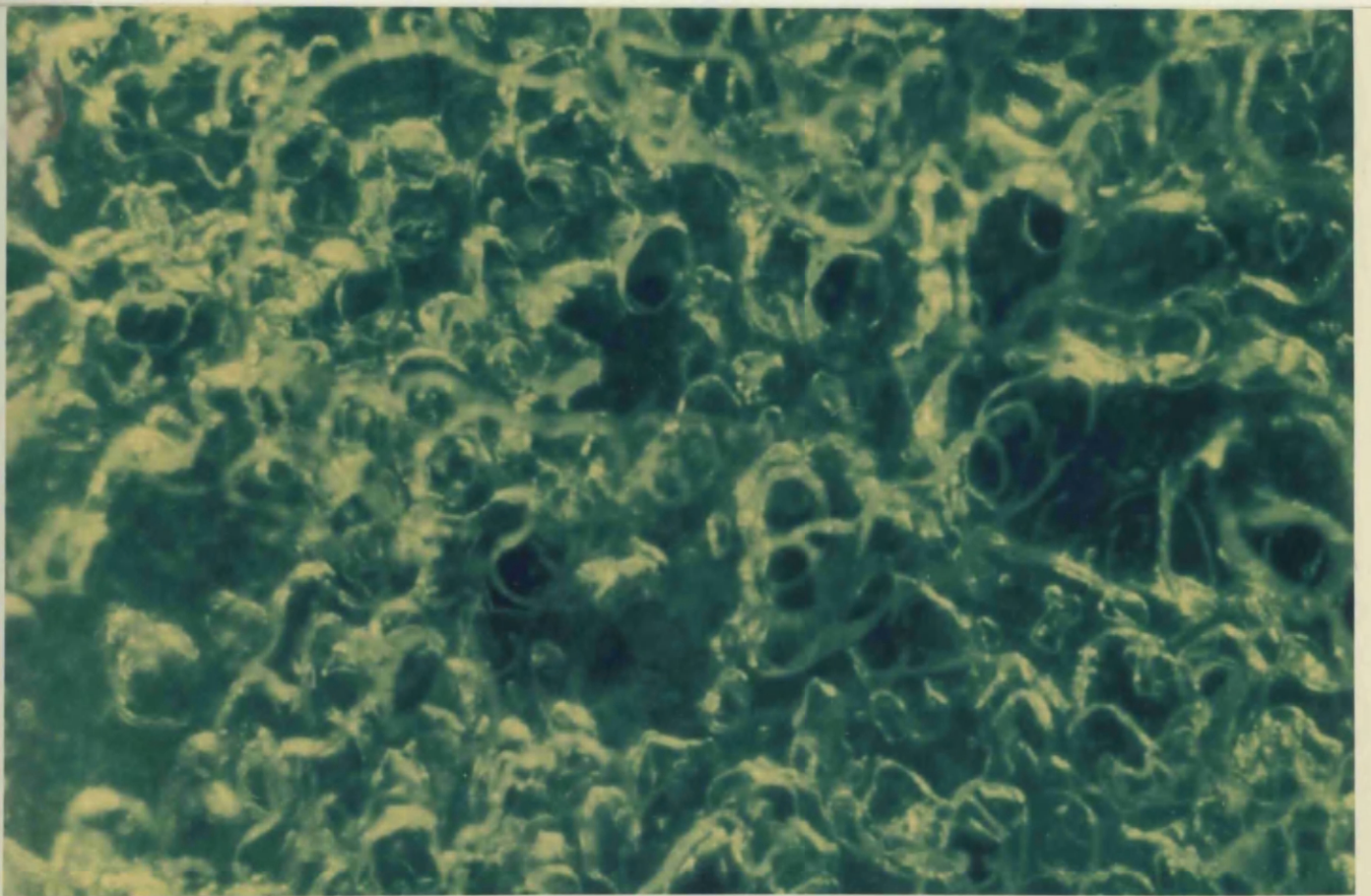


PLATE VIII

positively stained with the fluorochrome and lipochrome stains.

This contrasts with the findings of others (Boland & Klaus, 1964; De Sa, 1965) who found autofluorescence and positive fluorochrome staining after fixation with buffered formol-calcium. The formol-calcium solutions used in this study were not buffered and this may have influenced their effect on the alveolar surface. It is probable that the absence of fluorescence after rapid freezing with liquid N₂ and isopentane cooled with liquid N₂, was due to the tissue being allowed to warm up to -12°C before sections were cut. Ice crystals form at temperatures higher than about 40°C and cause disruption of the alveolar wall. In many cases, sections which had been stained with haemalum and eosin showed widespread disintegration of the alveolar cells, due to ice crystal formation. It is, therefore, not surprising that fluorescence was not seen in these cases. The possibility that the cooling of lung tissue to -170°C might have altered its fluorescent properties must also be considered. This postulate could not be investigated because it was not possible with the available equipment to cut sections of unfixed lungs at temperatures below those at which ice crystals form.

The autofluorescence seen in this study was similar to that described by Boland & Klaus and by De Sa on formol-calcium fixed lungs. Phosphine 3R and Rhodamine B have some specificity for lipids (De Sa, 1965). The positive reaction seen at the alveolar surface with these fluorochromes, suggests that there is a concentration of lipids here. The fluorescence however, might be due to the close apposition of fluorescing cell membranes of the type I alveolar lining cell; for it was noted that RBC outer membranes stained positively with these dyes. This finding is not new (Boland & Klaus, 1964; De Sa, 1965)

Staining with lipochrome dyes, such as phosphomolybdic acid, for choline containing lipids, and acid haematin, for phosphatides, suggests that there is a concentration of these lipids at the alveolar surface. The distribution of positive dye reaction was patchy, indicating either that the alveolar lipids are not uniformly

distributed, or that these dyes lack true specificity, or that processing had destroyed some but not all lipids. The first hypothesis seems the most likely. Staining by the PAS method which is specific for CHO groups, further identified the possible nature of the alveolar surface. The particular staining reaction seen in this study suggests that the alveolar surface contains neutral mucopolysaccharides. This leads one to speculate that there may have been a migration of mucus downstream into the alveoli, from the larger air passages, as first suggested by Niden, (1967).

Tracheal froth, produced during perfusion of the vascular bed with saline, has been shown to be surface active, (Bondurant & Miller, 1962). The thin fluorescent micelles seen in the tracheal froth, in this study, were of the same intensity and colour as the alveolar autofluorescence. One might be tempted to state that these micelles originated from the alveolar surface; but their stranded appearance was more characteristic of preformed filaments of organic material than the rolled up surface of alveoli. The fluorescence of these micelles was more labile than the alveolar autofluorescence, indicating either that the micelles did not originate from the alveolar surface or that there was a change in the nature of the lining material of the alveoli when it emerged in the tracheal froth.

The fluorescence of lung bubbles would support the hypothesis that they are derived from the alveolar surface. The presence of double fluorescent lines at the bubble surface, suggests that the bubbles have relatively thick walls; and further, that only those molecules on the internal and external margins of the bubble surface are excited by ultra-violet light; a physico-chemical explanation of this latter suggestion is not attempted. The solidity of these bubble walls is indicated by the persistence of fluorescence after the bubbles have been dried. This correlates with the finding that when lung bubbles are dried, a solid residue remains, (Pattle, 1965).

Hyperventilation had a variable effect on alveolar fluorescence, and virtually no effect on the fluorescence of lung bubbles. It has been shown earlier in this study that hyperventilation causes a marked change in the surface activity of intact lungs, as well as a significant change in the stability of lung bubbles. One can, therefore, conclude that while fluorescent techniques provide information about the nature of the alveolar surface and of lung bubbles, they in no way meet the demands of a quantitative estimate of pulmonary surface activity, nor do they resolve the problem of localising pulmonary surfactant. This subject is dealt with in PART II of this study.

PART II

MORPHOMETRIC STUDIES

"Unfortunately this is a field in which it is easy to produce numerical results, but difficult to decide what these results mean.

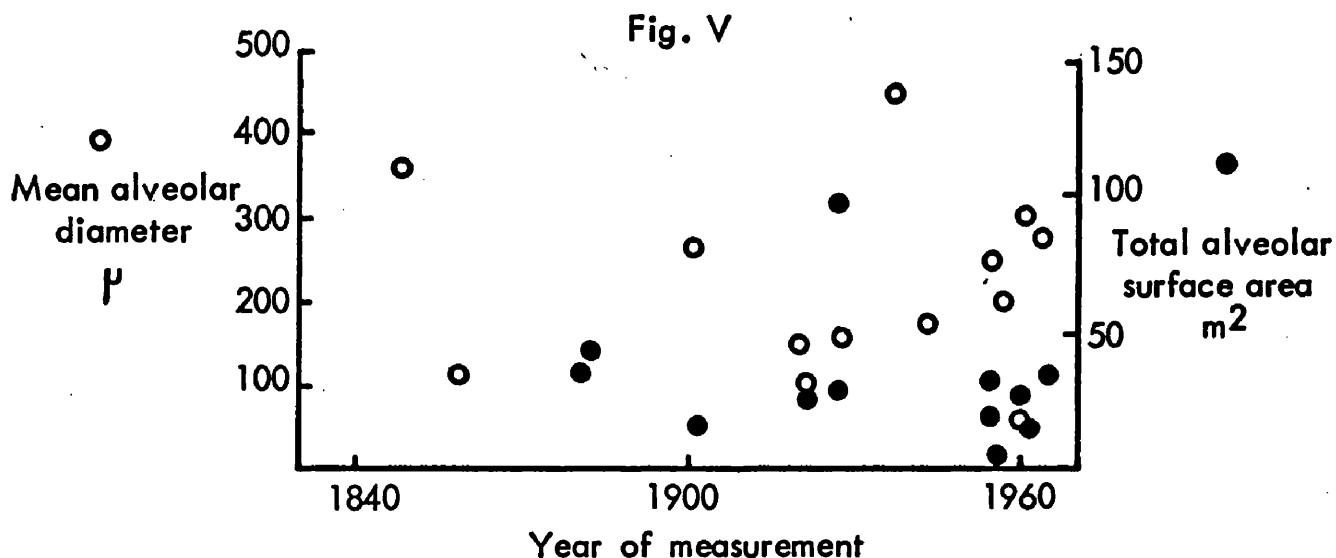
One way an investigator can help himself is to investigate the specimen by all possible methods at his disposal."

(Pattle, 1967)

MORPHOMETRIC STUDIES

INTRODUCTION

Lung morphometry is the precise measurement of the dimensions of pulmonary structures. Measurements of the size of pulmonary alveoli were made as long ago as 1731, by the Rev. Stephen Hales. He found that the average diameter of the alveoli of a calf's lung, was about 250μ ; from this he calculated that the total alveolar surface area was $27m^2$. Following these observations, there have been many measurements of the dimensions and total number of alveoli in human lungs. Fig. V illustrates how the results of measurements of the mean alveolar diameter and total alveolar surface area, have varied during the last 125 years.



It is obvious that with the passage of time, the variation in estimates has not diminished, despite the introduction of sophisticated methods of measurement and morphometric analysis.

The lung is a distensible organ: the extent to which the dimensions of its component structures change during the respiratory cycle, will depend on the relative mechanical properties of each component. The variation of results illustrated above, could be due to differences in 1. the degree of inflation,

2. the method of fixation and preparation of lung sections, 3. the methods of measurement, 4. the relative size of the lungs.

Tenney & Remmers (1963) have shown that in mammals there is a fairly constant relationship between body size and alveolar surface area. They also found that the ratio of total alveolar surface area to total oxygen consumption, was very nearly constant. The animals which they studied ranged from the shrew, with a body weight of 0.01 kg., to the whale, with a body weight of 5000 kg.

It is generally held that during lung inflation, the predominant volume change occurs in the alveoli and the alveolar ducts. Marshall (1962) has shown that when the lungs are fully inflated from a volume near functional residual capacity, there is a 60% increase in bronchial diameter and a 40% increase in bronchial length. The volume of the conducting airways accounts for only 10% of the total lung volume (Weibel, 1963a); so that the bulk of the volume change during lung inflation, occurs in the respiratory zone.

The size and the shape of the alveoli may change during various phases of the respiratory cycle (Dunnill, 1968). During quiet breathing at rest, the dimensional changes of alveoli may be minimal; but the changes occurring during deep inspiration and expiration are far from clear.

Keith (1901) stated that alveoli do not alter their volume or surface area during maximal inspirations. This view has been supported by Macklin (1929) who proposed that during deep inspiration, only the alveolar ducts expand, resulting in widening of the mouths of the alveoli; but without any significant change in alveolar volume. It follows from this concept that ventilation is served merely by volume changes occurring in the alveolar ducts and other airways upstream.

Functional studies indicate that the pulmonary diffusing capacity increases with increase in lung volume, (Cadigan, Marks, Ellicott, Jones &

Gaensler, 1961; McGraith & Thompson, 1959). The diffusing capacity of the lung is the volume of gas which diffuses per unit time per unit of pressure difference. It will depend on the nature and thickness of the diffusion barrier, as well as the area of contact. One can conclude therefore, that during lung inflation either the total diffusing surface increases and/or the diffusion barrier decreases.

Storey & Staub (1962) found in the lungs of cats, that the average diameter of both, alveoli and alveolar ducts, increased by 30% during maximum lung inflation, from functional residual capacity. They calculated that the volume of alveoli and of alveolar ducts would increase twofold, and that the total alveolar surface area would increase by 70%. Nonetheless, the mean alveolar diameter is of little geometric significance (Weibel, 1963a); since in any one lung at any moment of inflation, there is a profile of alveolar shapes and sizes. It has also been shown that in the lung of the erect subject there is a gradient of alveolar size, such that those at the apex are four times larger than those in the lower, dependent regions, (Glazier, Hughes, Maloney & West, 1967).

Dunnill (1967) measured the total alveolar volume and the total alveolar surface area in the lungs of dogs. He found that the alveolar volume and surface area were linearly related to lung volume. The ratio of the volume of the lung to its weight, was used as an index of the degree of inflation. Measurement of the dimensions of alveoli and alveolar ducts during lung inflation, while measuring the actual degree of inflation has hitherto not been done.

Pulmonary surfactant is thought to act only at the alveolar surface. Evidence has been produced earlier, indicating that hyperventilation results in a decrease in pulmonary surface activity. One suspects therefore, that a change in surface activity might be reflected in a change in the relative dimensions of alveoli and alveolar ducts.

The present study investigates the dimensional changes of alveoli and alveolar ducts at different degrees of lung inflation and following a period of hyperventilation.

PRINCIPLES OF MORPHOMETRIC ANALYSIS

GENERAL CONSIDERATIONS

The internal organization of tissues is most commonly studied on sections of fixed and embedded samples of tissue. For precise morphometric studies, appropriate sampling methods are necessary.

Random sampling is most efficient when applied to components which are themselves randomly, or homogeneously distributed. Whereas systematic sampling is most often used when the components are not randomly distributed.

Component distribution:

Biological structures are highly organised, so that randomness, as a basic requirement for most statistical methods, is, on the face of it, impossible to achieve. It is therefore, necessary to define how randomness of component distribution is interpreted in this study.

The spatial arrangement of the airways system of the lung is such that there exists a complex branching network of in series and in parallel units. The terminal unit, (a single alveolar duct with its associated alveoli) is in series with its parent respiratory bronchiole, and in parallel with other terminal units. On the other hand, there is no organised relationship between alveoli belonging to different terminal units; except to say that they occupy all available space.

A sample of parenchymous lung tissue therefore, contains alveoli which are randomly distributed, relative to one another. However, they are non-randomly distributed with respect to the entire lung; since the conductive elements of the airways and blood vessels are clearly, spatially orientated in an organised manner.

This concept has been summarised (Weibel, 1963b) and is set out below.

1. Random distribution of structures in space can be assumed if the units under investigation do not exhibit any stratified array in the unit tissue volume, even though they may be well organised into units of higher order.
2. Random distribution of structures refers only to a specific part of the tissue: alveoli are randomly distributed only with respect to the lung parenchyma.
3. When structures are not randomly distributed, a method of systematic sampling has to be adopted.

Tissue section thickness:

A tissue section is a very thin slice of tissue whose thickness can be reduced as far as is compatible with good optical resolution. Most morphometric procedures are carried out at relatively low magnifications. Provided the volume of an individual component greatly exceeds the thickness of a slice through it, the error will be negligible. Weibel (1963a) has derived a suitable correction for this error when the component volume relative to the section thickness is small: for spherical bodies this is given by

$$V'_{vi} = V_{vi} \cdot \frac{4r}{4r + 3h} \quad (\text{Equ. 3})$$

where: V'_{vi} is the actual fraction of the total volume which the component occupies,

V_{vi} is the measured volumetric fraction,

r is the radius and

h is the section thickness.

It can be seen that if h is very small the error becomes negligible.

A section can therefore, be regarded as a two dimensional sample of the tissue; thus confining morphometric methods to the measurement of lateral dimensions.

MEASUREMENT OF VOLUMES

The fundamental relation for the estimation of the volume of component structures was described in 1846, by the geologist Delesse. It is known as the Principle of Delesse. It states that the volume fraction, V_{vi} , of a component, i , in a tissue, can be estimated by measuring on a random section, the area fraction, A_{ai} , occupied by transections of i : so that

$$V_{vi} = A_{ai} \quad (\text{Equ. 4})$$

Methods of measurement:

Originally the only method of determination of the area fraction was by the laborious and inaccurate method of planimetry. There are now two commonly used methods.

Linear integration was first described in 1898 by Rosiwall. He found that the area fraction could be estimated by measuring the linear fraction of a test line which passes through component transections. He further showed that the test line need not be straight, as long as it did not have any bias to the underlying distribution of components.

Point counting was introduced by Glagoleff (1933) as a method of analysing the volumetric fractions of rock samples. He found that if a lattice of regularly spaced points was superimposed on a section of the sample, the fraction of points lying on the transected areas of the component equalled the area fraction. Thus the relation becomes,

$$\frac{P_i}{P_T} = P_{pi} = A_{ai} = V_{vi} \quad (\text{Equ. 5})$$

where: P_T is the total number of points on the lattice,
 P_i is the number of points lying on component i , and
 P_{pi} is the numerical point fraction.

The point counting method of estimating volumes has been shown to be statistically the most accurate method, (Hennig, 1959).

The absolute volume, V_i , of a component can be calculated if the total volume, V , and volume fraction, V_{vi} , of the tissue or organ is known:

$$V_i = V_{vi} \cdot V \quad (\text{Equ. 6})$$

ESTIMATION OF SURFACE AREAS

If a component has a well defined surface area, S_i , and is contained in a unit volume, V_n , their total surface area, S_i , can be defined as their surface density, S_{vi} :

$$S_{vi} = \frac{S_i}{V_n}$$

The surface of components appears on sections as contour lines, the length of which will be proportional to the surface density. If a test line of fixed length, L_t , is randomly placed in the tissue it will pierce through the surface of the components. The number of intersections, N_i , between the test line and the surface will be proportional to the surface density, S_{vi} , and to the length of the line, L_t .

Tomkeieff (1945) and Hennig (1956) independently derived the relation,

$$S_{vi} = \frac{2N_i}{L_T} = 2 \cdot N_{Li} \quad (\text{Equ. 7})$$

where: L_T is the total length of the line of n estimations and
 N_{Li} is the numerical fraction of the intersections.

Hennig also proposed that when the surface is considered as a double layer,

as in the case of the alveolar wall, the coefficient 2 in equation 7 should be replaced by 4.

The total surface area, S_i , of such a component in a given volume, V , is obtained from,

$$S_i = S_{vi} \cdot V = \frac{4N_i \cdot V}{L_T} \quad (\text{Equ. 8})$$

SURFACE TO VOLUME RATIOS

The surface to volume ratio of a tissue component is a useful measure of their geometric shape. Chalkey, Cornfield & Park (1949) derived a method of estimating surface to volume ratios from tissue sections. It is essentially a combination of the measurement of volumes by point counting, and of surface areas by linear intercepts. The surface to volume ratio, S_i/V_i , of individual components, i , is given by,

$$\frac{S_i}{V_i} = \frac{4N_i}{L_T \cdot P_i} \quad (\text{Equ. 9})$$

where: N_i is the number of intersections of component, i , by a test line of length L_T , and

P_i is the number of points lying on i .

TOTAL NUMBER OF COMPONENTS

Weibel & Gomez (1962) have shown that the estimation of the total number of components in a given volume, from thin sections requires a knowledge of the shape and distribution of the components. They have derived a dimensionless coefficient, β , which requires only an approximate knowledge of shape. When the coefficient β is known for a representative sample of the component shapes,

and the component volume fraction calculated by point counting analysis, the total number of the component, N_{vi} , is given by,

$$N_{vi} = \frac{1}{\beta_i} \cdot \frac{N_{ai}^{\frac{3}{2}}}{V_{vi}^{\frac{1}{2}}} \cdot K \quad (\text{Equ. 10})$$

where: N_{ai} is the number on a unit area of section, and

V_{vi} is the component volume fraction

K is a size distribution factor and is the ratio of the first to the second moment of distribution of the diameters of the components. Weibel & Gomez calculated that for a normal distribution of component diameters with a standard deviation of $\pm 25\%$, $K = 1.07$. For most purposes in biological work, the coefficient K can be assigned on arbitrary value of 1.02 to 1.1.

MEASUREMENT OF LINEAR DIMENSIONS

Measurement of lengths and diameters of randomly distributed components never corresponds to the characteristic dimension of their shape. Wicksell (1925) has analysed the probability of measuring the true radius of equal spheres which are randomly orientated within a containing volume, and also when a mixture of ellipsoids of various sizes is sectioned randomly, (Wicksell, 1927). He found that the arithmetic mean of the true diameters, \bar{r}_o , is equal to the harmonic mean of the apparent diameters, r_h , multiplied by $\pi/2$. The harmonic mean is the reciprocal of all measured diameters divided by the total number of diameters.

If a set of spheres of equal radius, r_o , is sections randomly, the relative frequency, ϕ_r , of the apparent diameters, r , is given by the expression,

$$\phi_r = \frac{r}{r_o} \cdot \int_0^R \frac{F_r dr}{\sqrt{r_o^2 - r^2}}$$

where: $F_r dr$ is the relative frequency of spheres, and
 R is the upper limit of diameters.

This equation can be reduced to the simpler form,

$$r_o = \frac{4\bar{r}}{\pi} \quad (\text{Equ. 11})$$

For an aggregate of spheres and ellipsoids of different sizes, the relative frequency of the apparent diameters ϕ_r is given by,

$$\phi_r = r \cdot \int_r^R f_r \cdot \frac{dr}{r_o \sqrt{r_o^2 - r^2}}$$

Thus the apparent diameter, r , of a spherical or ellisoidal body is taken as the geometric mean of the largest and the smallest diameters. The true diameter r_o is the geometric mean of the largest and smallest diameters in the central plane parallel to the plane of section. Knowing the frequency of apparent diameters ϕ_r one can calculate the frequency of true diameters, f_r , and by calculating the geometric mean, arrive at a value for the true diameter, r_o .

The error introduced by assuming a single shape distribution will in the case of the lung be relatively small, (Weibel, 1963b). Consequently in this study the

simple expression, $\bar{r}_o = \frac{4\bar{r}}{\pi}$ was used.

NORMAL TIDAL VOLUME AND FREQUENCY OF RESPIRATION IN GUINEA PIGS

The normal resting tidal volume and frequency of respiration were measured on unanaesthetized guinea pigs: so that accurate comparison of the effects of hyperventilation could be made. An attempt was made to correlate normal resting tidal volume and frequency of respiration with body weight.

STETHOGRAPHY

Two stethographs were constructed, (Plate IX). Each consisted of a fine bore polythene tube filled with mercury (internal diameter 0.5mm) and attached to a moulded perspex plate, fitted with an adjustable clasp. The ends of the tube were connected to a pair of copper terminals on the perspex plate. A 12v electrical signal was passed through the mercury column from a constant output device, (Type T/6, Roband Electronics, London). The signal from the stethograph was passed through a variable bridge circuit and recorded on a pen recorder.

One stethograph was placed around the lower part of the chest of the guinea pig, by wrapping the mercury filled tube around the chest and looping it over the adjustable clasp. The other was placed around the mid-abdomen.

The combined signal from both stethographs was used as an index of the total respiratory excursion.

PROCEDURE

Six guinea pigs whose body weights varied from 350 - 800g, were used for measuring resting tidal volume and frequency of respiration. The stethographs were placed on the animals, so as to cause the minimum of discomfort. The stethograph terminals were connected to the constant output device and the stethograph signal recorded. The animals were returned to their cages and left

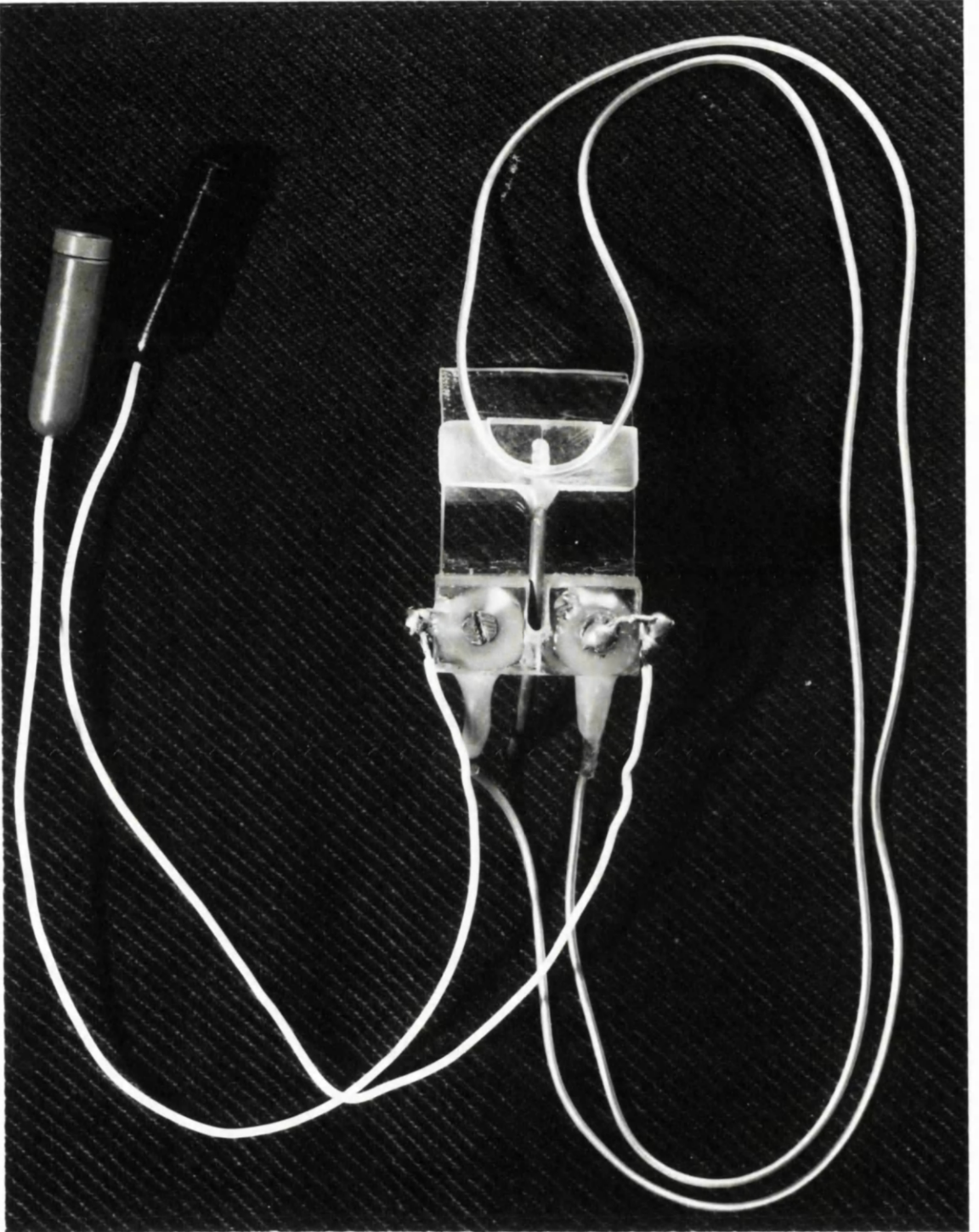


PLATE IX - STETHOGRAPH

undisturbed for at least 20 min. A record of the resting frequency and tidal volume was then taken.

Calibration of the stethograph:

The signal from the stethograph depends on the resistance of the column of mercury which in turn depends on the internal diameter of the column.

Calibration of the stethograph against an independent method of measuring tidal volume enabled the stethograph to be used to give an absolute measure of tidal volume.

One guinea pig was anaesthetized with intraperitoneal pentobarbitone sodium (dose 60mg/kg), a tracheostomy was performed and the trachea cannulated. The stethographs were placed on the animal which was then ventilated with a Palmer small animal pump. Tidal volume was measured by pneumotachography, using a miniature flow head (Fleisch), placed in the tracheal cannula (Plate XII) The differential pressure signal from it was measured by a Greer electromanometer. Integrated volume was obtained by passing the output signal from the electromanometer through an integrator (Mercury Electronics) and recorded on a pen recorder.

The stroke volume of the pump was varied from 2 - 50 cc and the frequency from 20 - 170 strokes per minute. Integrated volumes were compared with the stethograph signal.

RESULTS

The stethograph signal varied linearly with the tidal volume; but at high tidal volumes (45 cc) when a high frequency was used (100/min.), the stethograph signal became erratic. This was due to disruption of the column of mercury connection with the copper terminals. Unsuccessful attempts were made to prevent this occurrence by altering the design of the terminals.

The resting tidal volumes and breathing frequencies were small compared with those used for the calibration of the stethographs; so that the design error mentioned above proved to be insignificant.

The resting tidal volumes of all six guinea pigs were fairly constant within the range 2.5 - 3.5 cc. The resting frequencies of respiration ranged from 20 - 46 breaths per minute. Poor correlation between body weight and these values was found.

	Correlation coefficient	Significance level
Tidal volume/body weight	0.68	P' = 0.1
Frequency/body weight	0.62	P' = 0.05

DISCUSSION

Stethography was a convenient way of measuring resting breathing frequency in the guinea pig. The measurement of tidal volume was less convenient by this method, but still fairly accurate. The lack of an adequate statistical correlation between tidal volume or frequency of respiration against body weight was probably due to the small number of animals used.

Kleinman & Radford (1964) found that, for all mammals, the tidal volume (V_t) in cc frequency (f) in breaths/min, and body weight (W) in g, followed the relation,

$$V_t = \frac{W^{0.74}}{(1 - k) \cdot f} \quad (\text{Equ. 12})$$

where k is a coefficient relating dead space and tidal volume.

The value of k for guinea pigs is 0.35 (Mead, 1960). Introducing this value into equation 12 gives,

$$V_t = \frac{W^{0.74}}{0.65 f} \quad (\text{Equ. 13})$$

The values of f , calculated from this equation, using experimentally determined values for V_t , corresponded closely with the measured values of f . It was therefore, decided that in subsequent experiments involving artificial ventilation, a constant value of 3 cc V_t would be assumed, and the value for f calculated for the weight of each animal, according to equation 13.

LUNG VOLUMES AND DEGREE OF INFLATION

The collected data from 22 guinea pigs, 5 rats and 15 mice was used to establish relationships between body weight and each of the following:

1. wet lung weight, 2. collapsed lung volume, and 3. the total volume of the lungs.

Wet lung weight was measured immediately following removal of the lungs from the animal. The lungs were dissected out and mediastinal fatty tissue separated. Thus wet lung weight comprised the weight of both lungs, both main stem bronchi and the lower 1/3 of the trachea.

Collapsed lung volume (cV) was measured by saline displacement after the lungs had been allowed to collapse slowly. It was therefore, the volume of the lung tissue plus a volume of trapped air. Standardising the technique of collapsing the lungs, reduced this variable to a minimum.

Total volume of the lungs (TV_L) was taken as the volume of the lungs at which the inflation pressure started to rise steeply, during a pressure-volume manoeuvre, (see page 40 for details of the method). It represents the maximum inflation volume of the lungs.

Degree of inflation was the volume of the lungs at any given moment of inflation, expressed as a percentage of the total volume of the lungs.

RESULTS

Regression analysis was performed on the values for TV_L , cV and wet lung weight as functions of body weight. All three parameters were found to be linearly related to body weight. Table V lists the correlation coefficients and regression equations for each species and for the combined group. All correlations were highly significant; for the rats each parameter was significant at $P < 0.05$,

for the mice and the guinea pigs and for the combined group, each parameter was significant at $P < 0.001$.

Analysis of variance was performed on the guinea pig data relating TV_L to body wt. It was found that the limits of error of TV_L were ± 1.73 cc.

Fig. VI shows the plots of TV_L , cV and wet lung wt. against body wt. for the whole group, (broken lines) for for guinea pigs alone, (solid lines).

The upper curve relating TV_L to body wt. was used for the prediction of TV_L in subsequent experiments where its measurement was impossible. The error introduced by making this prediction was insignificantly small, since the limits of error of the measured data was less than 3% of the absolute values.

	MICE n=15	RATS n=5	GUINEA PIGS n=22	COMBINED n=42
WET LUNG wt / B. wt.	$r = 0.8142$ $y = 0.0076x + 0.0079$	$r = 0.7842$ $y = 0.141x - 1.02$	$r = 0.9007$ $y = 0.0074x + 0.0098$	$r = 0.9892$ $y = 0.0073x + 0.018$
cV / B. wt.	$r = 0.9470$ $y = 0.0066x + 0.206$	$r = 0.8351$ $y = 0.014x - 0.167$	$r = 0.9374$ $y = 0.015x - 1.149$	$r = 0.9624$ $y = 0.009x - 0.164$
TV _L / B. wt.	$r = 0.9112$ $y = 0.0946x + 1.008$	$r = 0.7685$ $y = 0.178x + 0.328$	$r = 0.9056$ $y = 0.106x - 1.375$	$r = 0.9863$ $y = 0.11x - 1.234$

TABLE V

Correlation coefficients (r) and linear regression equations of WET LUNG wt./ COLLAPSED LUNG VOL. (cV)/TOTAL VOL. OF THE LUNGS (TV_L), denoted by, y , against BODY WEIGHT (B. wt.) denoted by, x . All values of r significant at $P = 0.01$

METHODS OF PREPARATION OF LUNGS FOR MORPHOMETRIC ANALYSIS

INTRODUCTION

The lungs of 23 anaesthetized guinea pigs were excised and examined microscopically after one of the following periods of artificial ventilation: 2 breaths, 5 min, 10 min, 15 min. A technique was developed for the rapid freezing of the lungs precisely at the end of the ventilation period, and at a predetermined point on the respiratory cycle.

The degree of lung inflation was calculated from the volume of the lungs at the instant just prior to freezing, and was expressed as a percentage of the predicted total volume of the lungs (see page 77)

Two further guinea pigs were included in this study for electronmicroscopic examination of the lungs. The lungs of one of the animals were examined fully inflated, while those of the other animal were examined when collapsed.

RAPID FREEZING TANK-RESPIRATOR

A technique was developed by which guinea pigs could be artificially ventilated with a positive and negative cycle, and rapidly frozen at the instant of cessation of ventilation.

A tank-respirator was constructed (Plate X) consisting of an upper chamber made from $\frac{1}{4}$ inch thick perspex and a lower chamber made from $\frac{1}{4}$ inch copper plate. A trapdoor with an external release mechanism, separated the two compartments. The lower chamber which contained isopentane, was suspended in a second copper chamber, containing liquid N_2 at $-170^\circ C$. These were insulated with 1 inch thick polystyrene blocks. The whole assemblage was housed in a large wooden box (Plate XI and fig. VII).

Liquid N_2 was continuously fed into the lowermost copper chamber

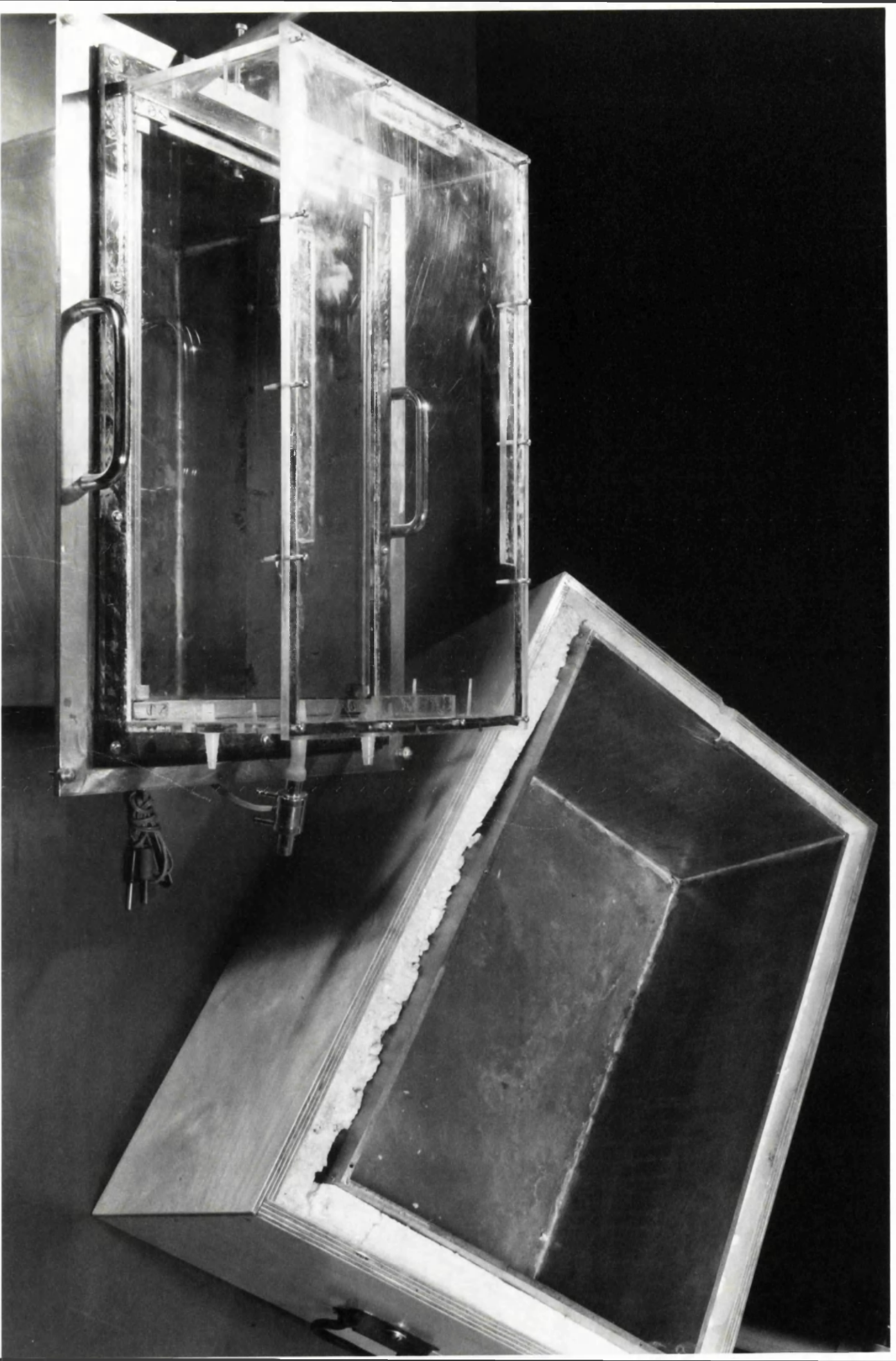


PLATE X - RAPID FREEZING TANK-RESPIRATOR

RAPID FREEZING TANK RESPIRATOR .

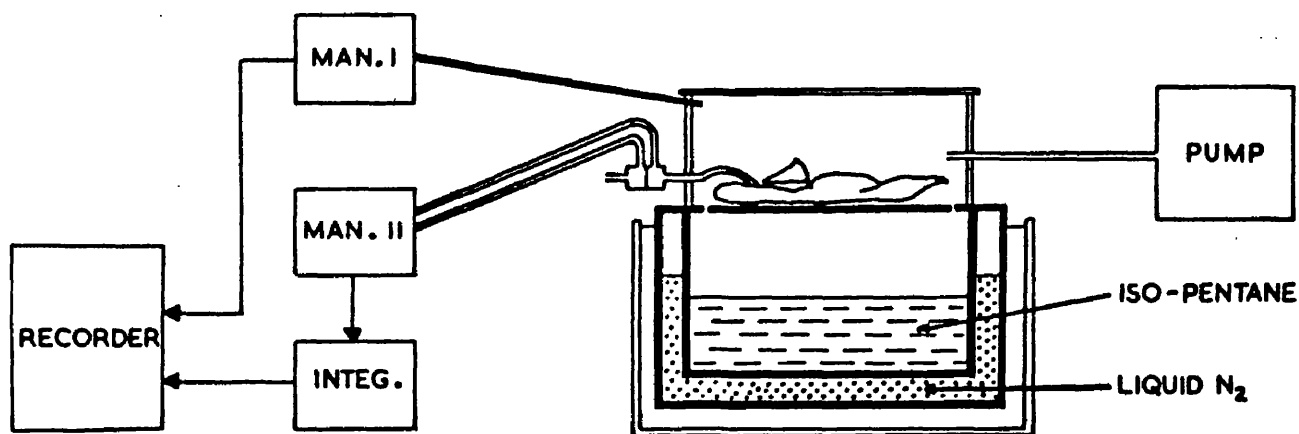


Fig. VII

through a high pressure valve tap, fitted to a liquid N_2 canister. The upper perspex chamber could be sealed and connected to a cyclical positive and negative pressure pump.

Recording of ventilation pressure and tidal volume: (Plate XIII)

Two small perspex tubes were screwed through the wall of the upper chamber of the tank-respirator. One was connected to a Greer electromanometer so the pressure within the chamber, when sealed, could be measured. A miniature flow head (Fleisch) (see Plate XII) was fitted to the outside of the other tube, while a tracheal cannula was connected to this tube inside the chamber, for the measurement of tidal flow.

Differential flow head pressure was measured by a Greer electromanometer and integrated (Mercury Electronics Integrator), to give a measure of tidal volume. The signal from the integrator was amplified by passing it through a step down resistance box. Tidal volume and tank-respirator pressure were recorded on a pen recorder (YEW x, x-y pen recorder).

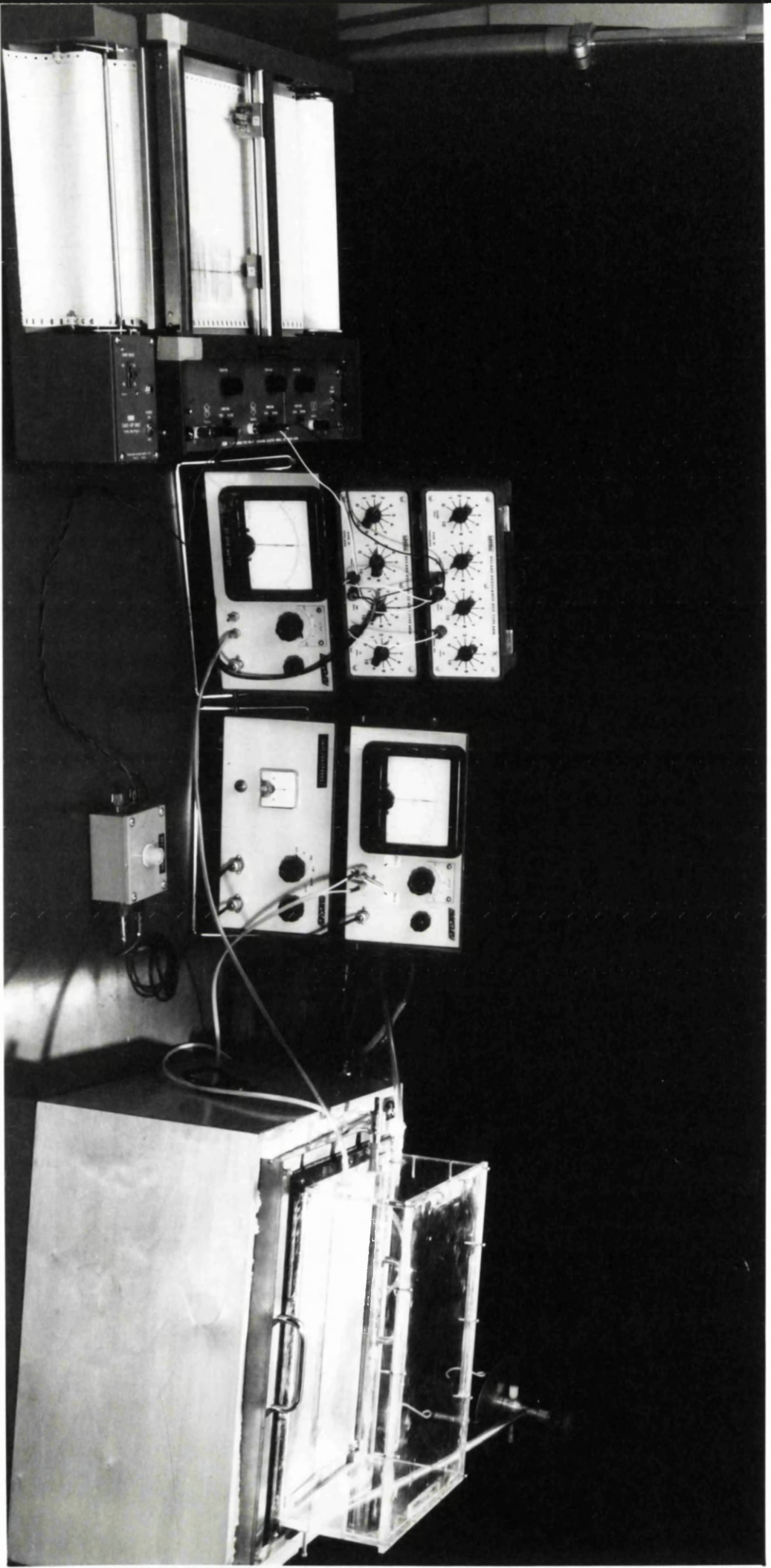


Fig. XI - RAPID FREEZING TANK-RESPIRATOR, EXPERIMENTAL LAY OUT

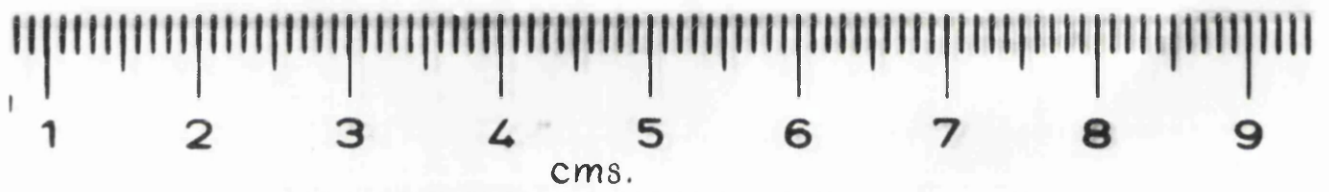
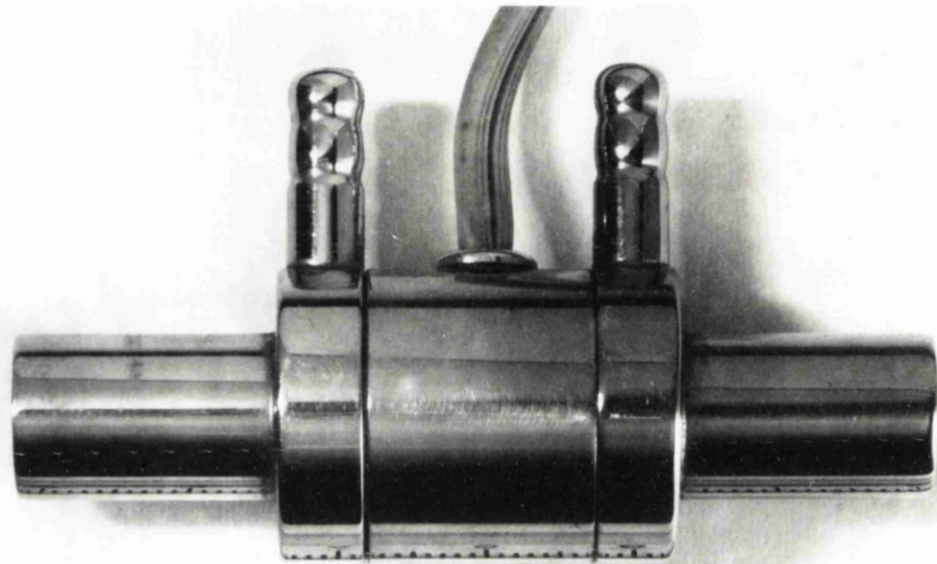


PLATE XII - FLOW HEAD

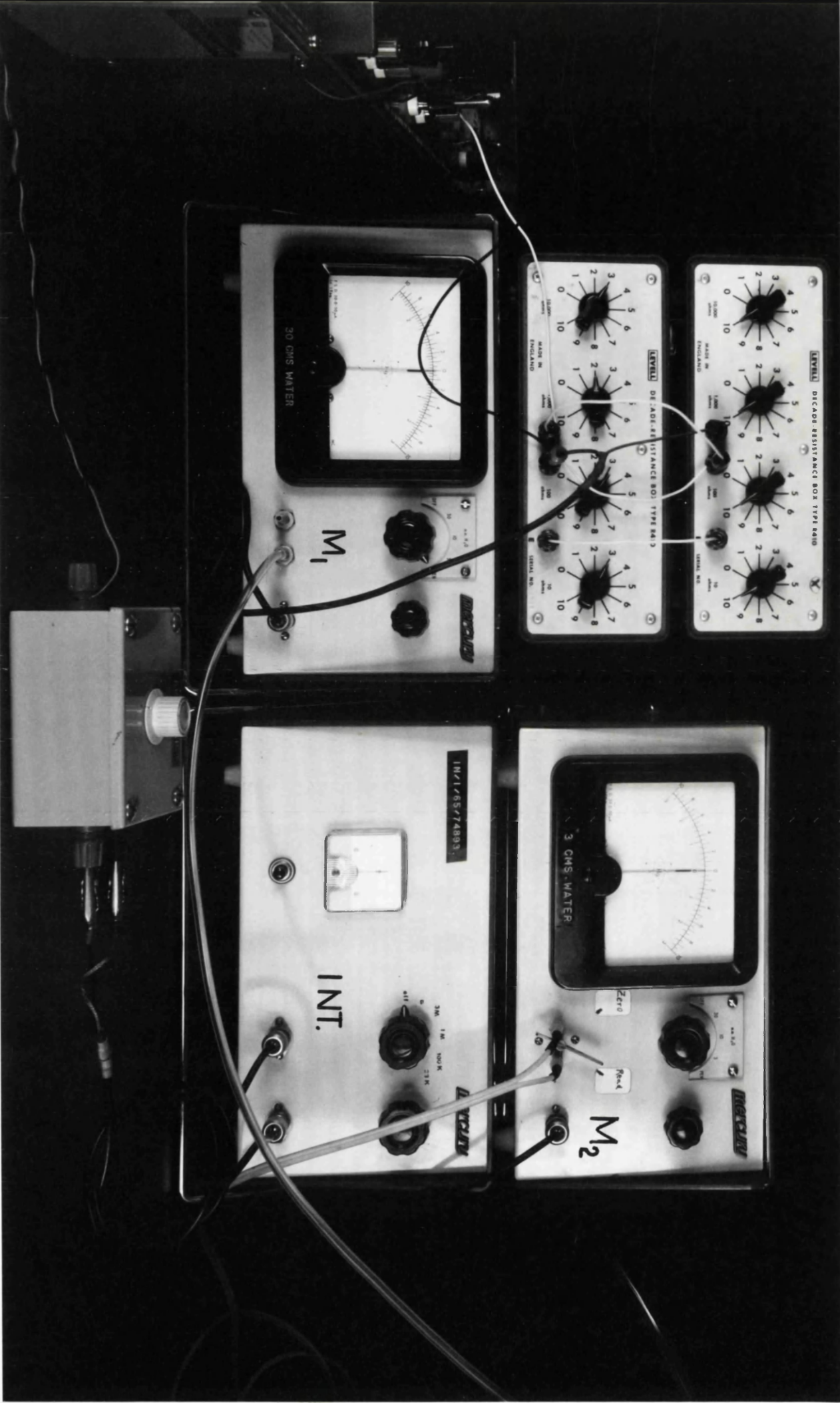


PLATE XIII - INSTRUMENTATION FOR MEASURING TIDAL FLOW AND TANK PRESSURE

- M₁ - MANOMETER 1 (pressure)
- M₂ - MANOMETER 2 (Flow)
- INT - INTEGRATOR

PROCEDURE

23 guinea pigs were anaesthetized with intraperitoneal pentobarbitone sodium (dose 60 mg/kg) and weighed. A tracheostomy was performed and the trachea cannulated. The tracheal cannula was connected to the flow head and recording of tidal flow set up. The sternum was split and the anterior chest walls widely separated. The volume of air expired when the lungs collapsed after opening the chest, was recorded.

The animals were placed in the tank-respirator above the rapid freezing chamber, which was then sealed. Artificial ventilation was immediately begun. The interval between thoracotomy and commencement of ventilation was less than 30 seconds.

Each animal was assigned, with the aid of a latin square, to one of four ventilation groups. These were 1. controls (period of ventilation, 2 breaths), 2. 5 min ventilation, 3. 10 min ventilation, 4. 15 min ventilation. The frequency of ventilation was set at a constant 32 strokes per minute.

The negative chamber pressure used for artificial ventilation was in all cases, that which would inflate the lungs to the predicted total volume of the lungs (page 77). Initially this was achieved by trial and error. In most animals studied it was found to lie between -30 and -40 cms H_2O . The positive chamber pressure used was in all cases 3 cms H_2O : this proved sufficient to collapse the lungs.

Thus all animals were artificially ventilated with a volume equal to the total volume of the lungs, minus the volume of lung tissue (collapsed lung volume).

Precisely at the end of the ventilation period, and at a predetermined point on the respiratory cycle (i.e. low lung volume, $\frac{1}{3}$ inflated, $\frac{2}{3}$ inflated, or fully inflated) three things were done simultaneously.

1. The airway was clamped.
2. The trapdoor separating the upper and lower chambers of the tank-respirator was released.
3. The respirator pump was switched off.

The animal immediately dropped into the rapid freezing chamber containing isopentane cooled to -170°C by the liquid N_2 in the outer chamber. The fall was broken by a thin polystyrene sheet floating on the surface of the isopentane.

The animal was left in the isopentane for about 15-20 minutes, by which time it had frozen completely solid. During this time the tank-respirator was unsealed.

Samples of the lower lobes of both lungs were obtained by chipping the lungs with a bone chisel and a pair of bone forceps, which had been cooled in liquid N_2 . The lung samples were rapidly transferred to a solution of fixatives (see below) cooled to -70°C by solid CO_2 (Drikold, I.C.I.).

FREEZE SUBSTITUTION METHOD OF FIXATION

Freeze substitution is the fixation and hydration of frozen tissues while they are maintained in the frozen state. If a tissue is frozen rapidly enough, the structural relationships will be preserved as long as the temperature does not rise above the critical level (-30 to -50°C) at which ice crystals grow. The frozen tissue therefore, must be fixed and dehydrated at a temperature below -50°C . The fixative solution must retain its fixating properties at these temperatures and in order that dehydration can occur they must be dissolved in non-aqueous media. When fixation and dehydration are complete the specimen can then be warmed to room temperature without disturbing the tissue architecture.

The fixative solution used was a modification of that described by Storey &

Staub (1962). It consisted of a stock solvent solution of ABSOLUTE ALCOHOL 75%, ACETIC ACID 5%, ACETONE 25%. The following were freshly made up in the stock solvent solution: POTASSIUM DICHROMATE 2%, PICRIC ACID 3%, MERCURIC CHLORIDE 4%.

The working strength of the fixative solution was obtained by diluting it, by 1 in 3 in the stock solvent solution.

Procedure:

1. Samples of lung tissue were rapidly and completely frozen in isopentane cooled to -170°C by liquid N_2 .
2. These were transferred to the fixative solution precooled to -70°C by solid CO_2 ('Drikold', I.C.I.).
3. The specimens were stored in a deep freeze at -60°C for 3-4 weeks, until fixation and dehydration were complete. Every 3 days during this time the fixative was changed for freshly made up solution.
4. When fixation was complete, the specimens were transferred to absolute alcohol precooled to -60°C . They were brought slowly up to room temperature, and transferred to 70% alcohol.
5. The specimens were embedded in low melting point paraffin wax.

Preparation of microscope sections:

Serial sections were cut on a sliding microtome at a thickness of 5μ and run onto warm water. The sections were picked up serially onto a clean glass slide, 6 sections per slide. They were then brought down through the alcohols to xylol, stained with haemalum and eosin and mounted in Canada Balsam.

SAMPLING METHODS

Primary sampling was carried out on 10 slides each containing 6 sections from each lung sample. One section per slide was chosen randomly, giving a total of 10 sections per lung sample.

Secondary sampling was carried out on each of the sections selected by

primary sampling. The sections were viewed with a light microscope fitted with a movable stage. The movable stage had horizontal and vertical controls calibrated by two vernier scales. The pleural edge was centred in the viewing field. Five fields were then chosen 'blind' by moving the vernier scales by random increments. The fields were then photographed; on each film a stage micrometer was also photographed as a magnification check. Photographic enlargements were made of the lung sections and the stage micrometer. Morphometric measurements were made from these enlargements.

Thus each lung specimen yielded 50 fields for measurement. Each field was further subdivided into 40 equal areas by superimposing a grid over the photographs. This gave a total of 2000 areas of measurement, for each lung specimen. By using such a large number of measuring areas the relative error of the methods of measurement was reduced to an insignificant factor, (Weibel, Kistler & Scherle, 1966).

Areas which contained conducting airways and large pulmonary blood vessels were rejected from analysis. Thus measurements were confined to the respiratory portion of the lungs.

CORRECTION FOR SHRINKAGE DUE TO PROCESSING OF THE LUNGS

VOLUME SHRINKAGE DUE TO RAPID FREEZING FOLLOWED BY FREEZE SUBSTITUTION

Volume measurement by saline displacement:

The volume of the lungs was measured by saline displacement in a modified Archimedes jar (Plate XV.). Displacement of saline caused a pressure change in the side reservoir; this was measured by a Greer electromanometer and recorded on a pen recorder. A step down resistance box was constructed to amplify the signal from the electromanometer. This enabled very small volumes to be measured accurately. It was necessary to dampen the effects of vibration because of the increased sensitivity; this was done by placing a constriction in the tube connecting the side reservoir to the Archimedes jar and by resting the jar on thick shock absorbent foam (Fig. VIII).

The accuracy of the method was tested using brass cubes whose volumes were exactly known. The volumes of three brass cubes (1 cc, 8 cc & 27 cc) were measured fifty times each by saline displacement. The standard deviation of these measurements were for each of the brass cubes, ± 0.016 , ± 0.084 , ± 0.172 respectively; the standard errors of the means were ± 0.0023 , ± 0.012 , and ± 0.0246 cc.

Procedure:

15 mice, 5 rats and 18 guinea pigs were anaesthetized with intraperitoneal pentobarbitone sodium (dose, 30 mg/kg, 40 mg/kg, 60 mg/kg respectively). A tracheostomy was performed and the trachea cannulated. The chest was opened by splitting the sternum. The lungs and trachea were removed intact and weighed. The collapsed lung volume was measured by saline displacement. The total volume of the lungs was measured by performing a pressure-volume manoeuvre (see page 40

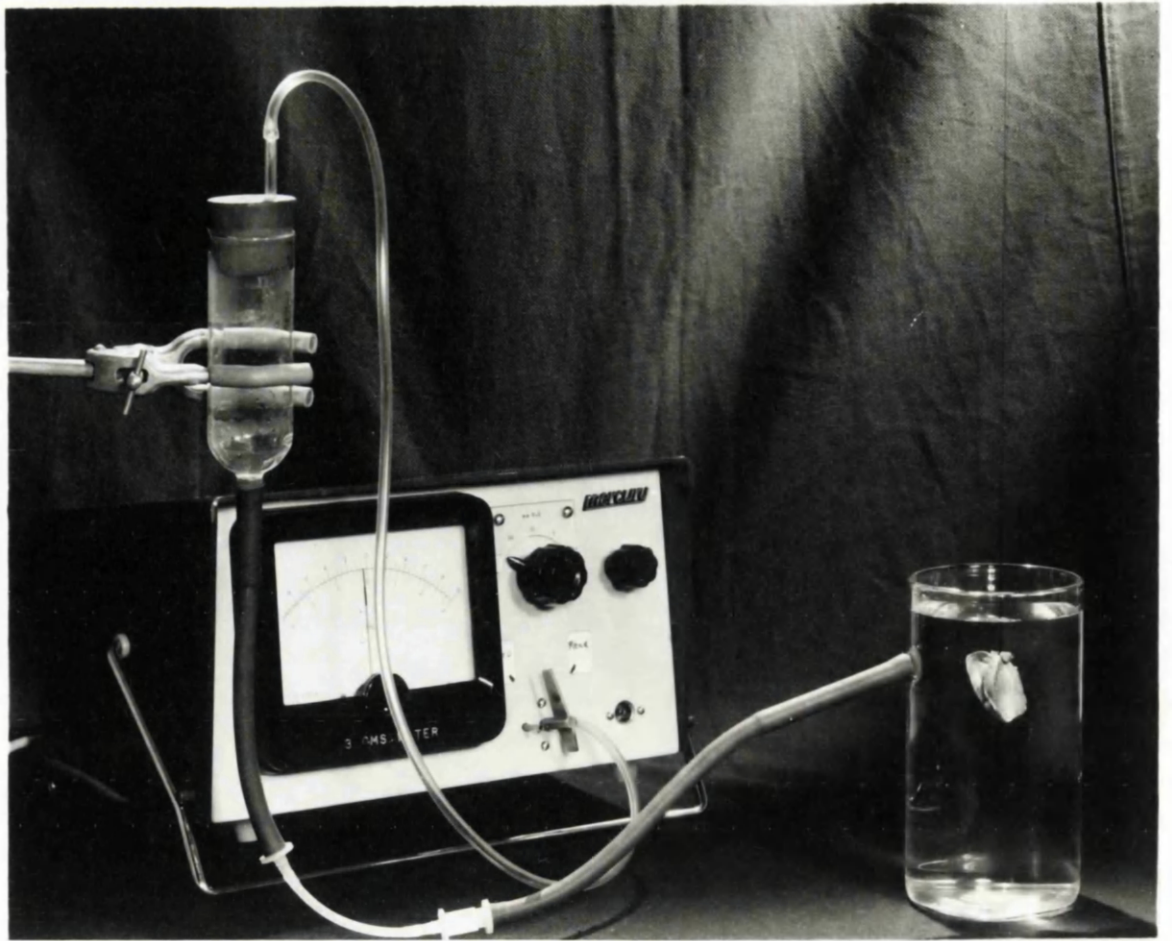


PLATE XIV - VOLUME MEASUREMENT BY FLUID DISPLACEMENT

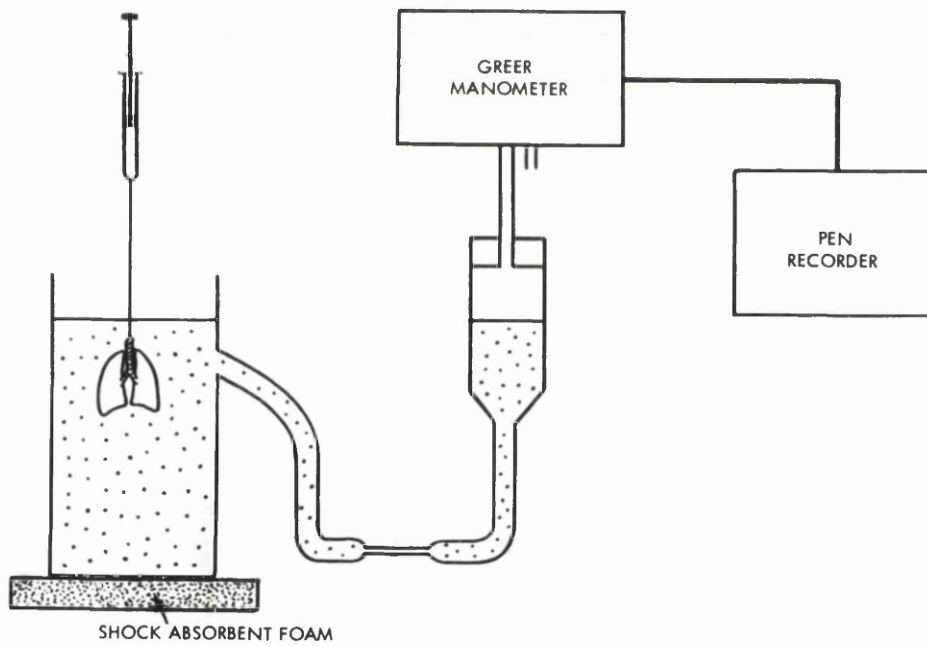


Fig. VIII

VOLUME MEASUREMENT BY FLUID DISPLACEMENT

for method). The lungs were then allowed to collapse or were inflated with air and the trachea tied.

The lungs were again weighed and their volume measured by saline displacement. They were immediately immersed in isopentane cooled to -170°C by liquid N_2 , until they were completely frozen, then quickly transferred to the fixative solution for fixation by the freeze substitution method (see page When fixation was complete, the lungs were brought up to room temperature and placed in 70% alcohol. The post-fixed volume of the lungs was measured by displacement in 70% alcohol. The degree of inflation was taken as the displaced volume of the lungs just prior to rapid freezing and expressed as a percentage of the total displaced lung volume. Total displaced lung volume equalled the total volume of the lungs, obtained by performing a pressure-volume manoeuvre, plus the collapsed lung volume, measured by saline displacement.

RESULTS

The results of measurements on the lungs of mice and rats were essentially similar to those made on the lungs of guinea pigs; although the absolute values for shrinkage differed. Only the results obtained from the guinea pigs lungs will be mentioned in detail; since this was the species used in subsequent experiments where shrinkage factors were applied.

The measured shrinkage was found to depend on the volume of the lungs at the time of freezing. Inflated lungs had greater shrinkage than collapsed lungs (Fig. IX).

The percentage shrinkage of collapsed lungs was fairly constant at a value of $15\% \pm 3.43$ (SEM). In lungs which were inflated the percentage shrinkage depended on the degree of inflation (Fig X). The relationship was found by the method of least squares to fit a hyperbolic function (regression equation $y = 3.7(x - 15)^{0.35} + 13$ where y is % shrinkage and x is degree of inflation). As the degree of inflation

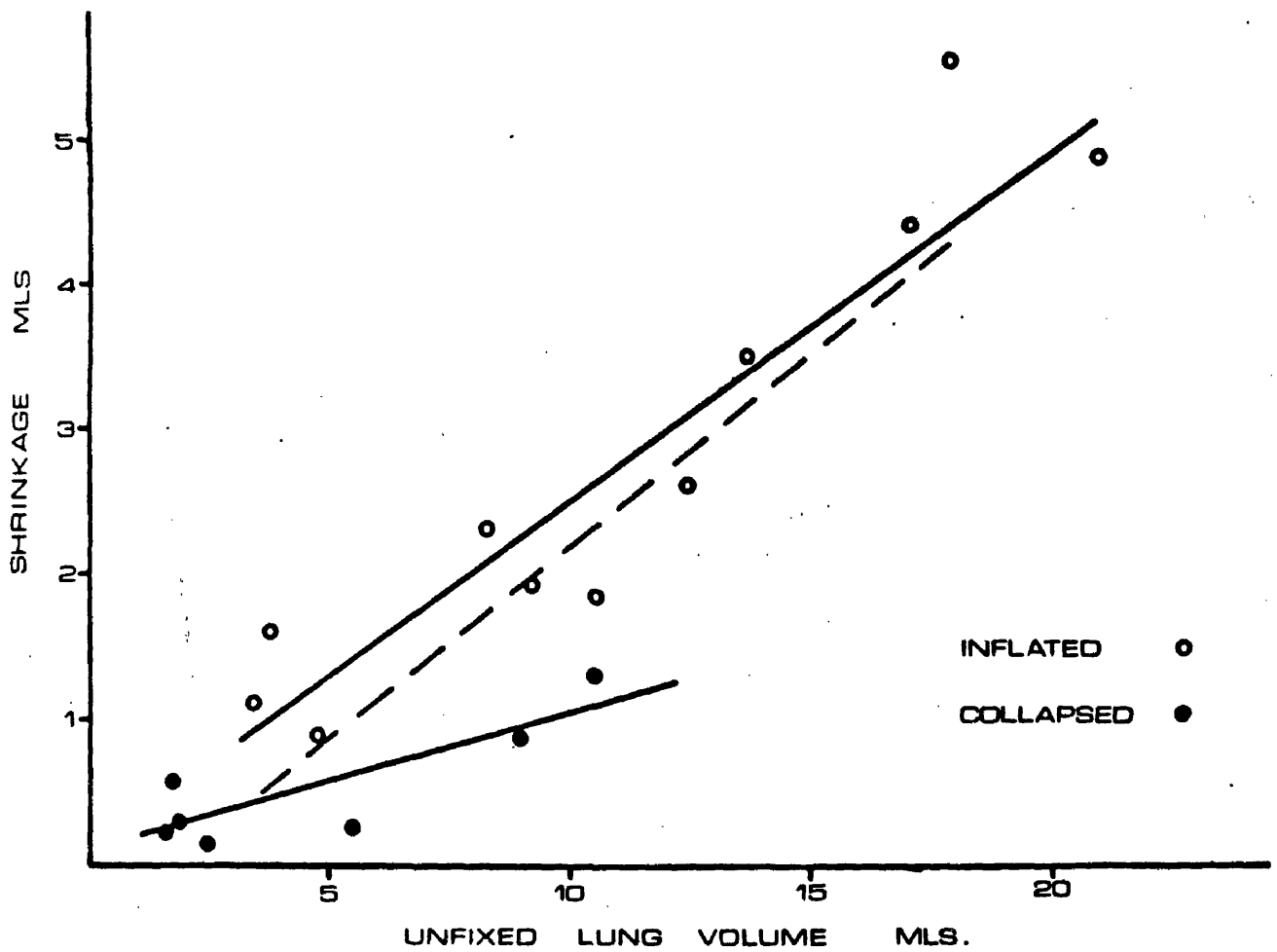


Fig. IX. Measured shrinkage volume related to the volume of the lungs before fixation.

Solid lines: guinea pigs

Collapsed lungs - $y = 0.938x + 0.0897$
 $r = 0.8392 (P' = 0.01)$

Inflated lungs - $y = 0.236x + 0.1187$
 $r = 0.9202 (P' = 0.01)$

Broken lines: theoretical relationship based on a mathematical model (see text on page 87.)

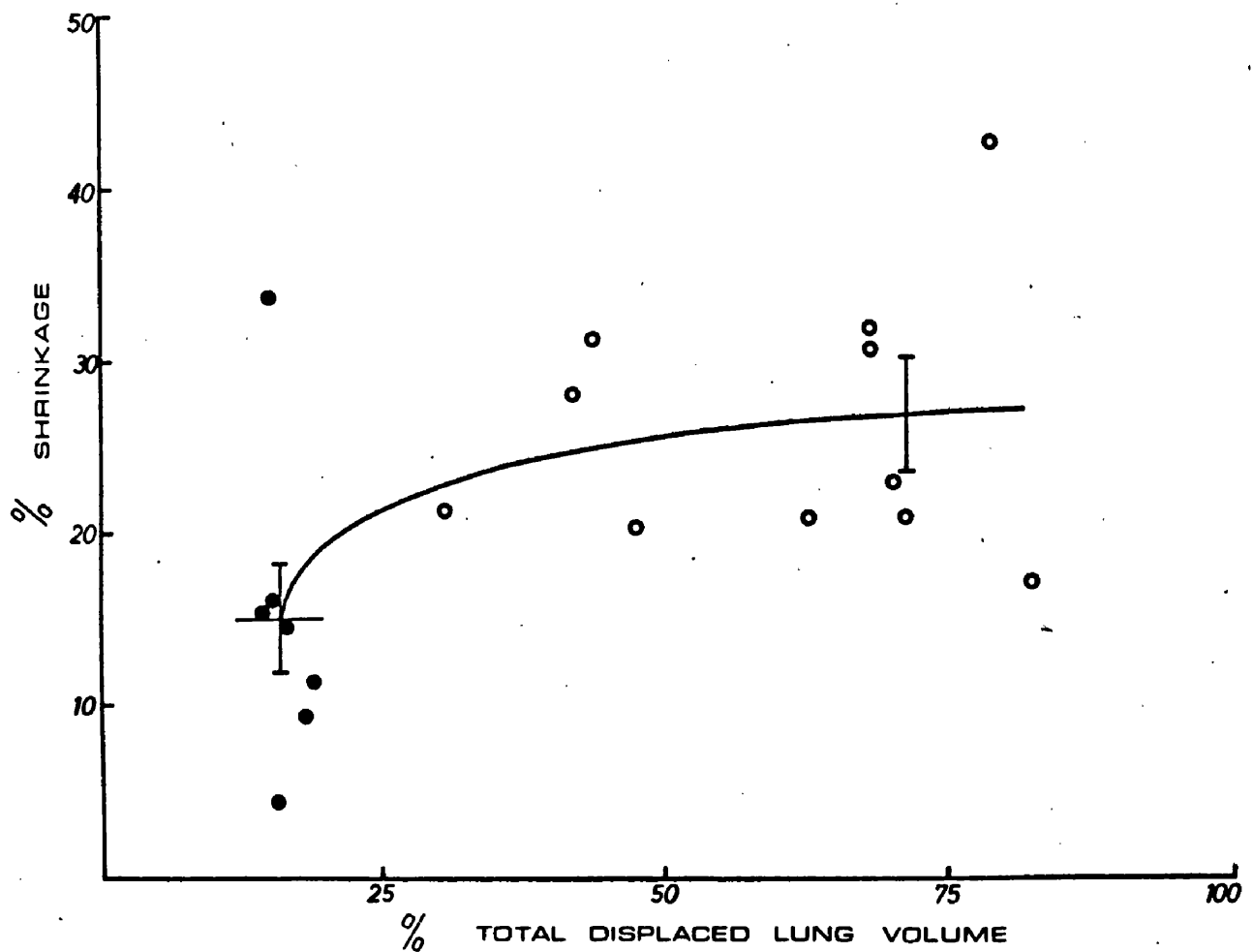


Fig. X. Percentage shrinkage related to the degree of lung inflation (measured as a percentage of total displaced lung volume) for guinea pigs.

Collapsed lungs - solid ●
 Inflated lungs - open ○

The mean shrinkage ± 1 SEM is shown for the collapsed lungs and for lungs inflated to 60-80% total displaced lung volume. The difference between the means is significant at $P = 0.001$. The curve shown follows the function $y = 3.7(x - 15)^{0.35} + 13$, where $y = \% \text{ shrinkage}$ and $x = \% \text{ total displaced lung volume}$.

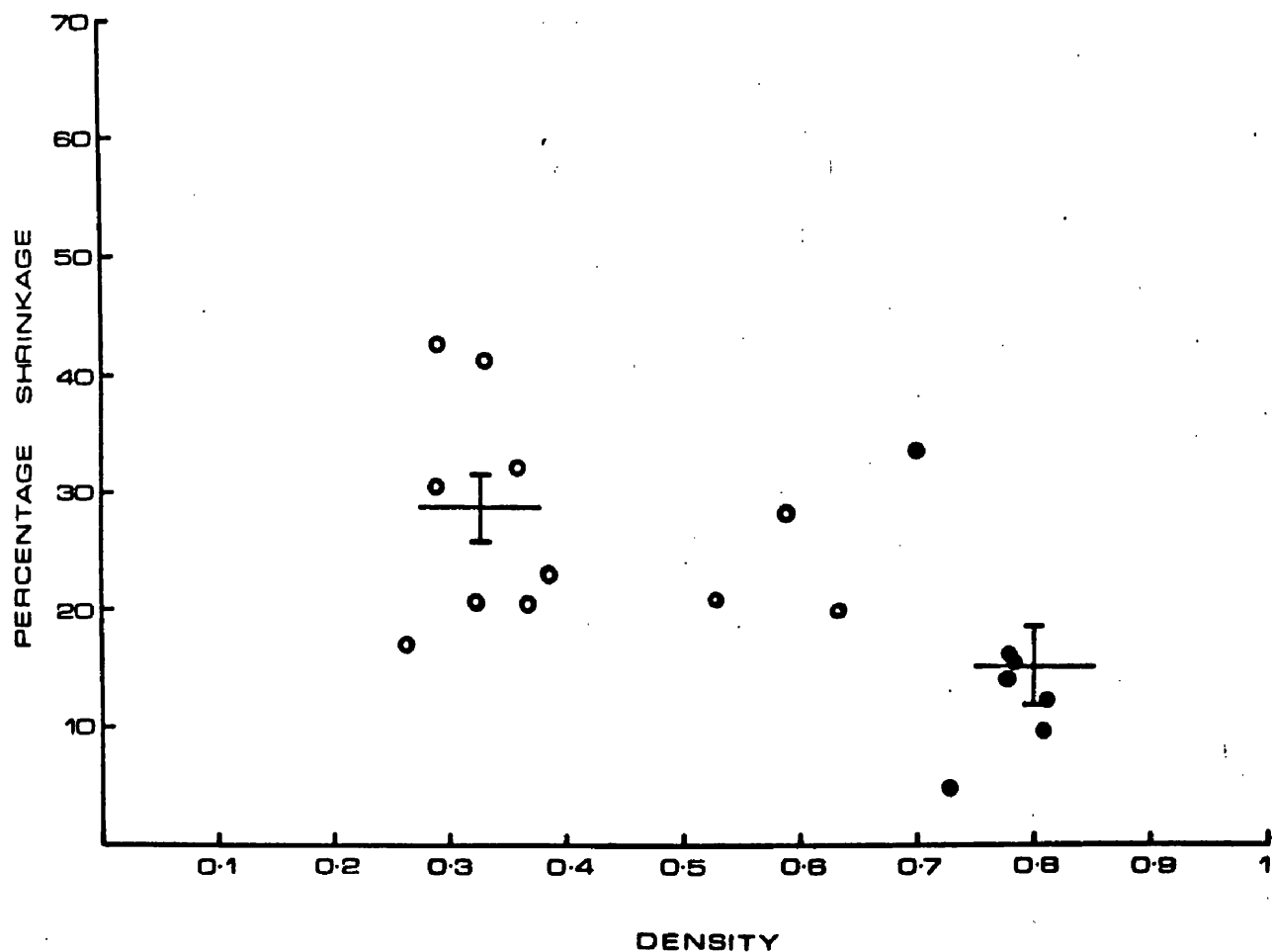


Fig. XI. Percentage shrinkage related to the density of the lungs of guinea pigs at the moment of freezing.

Collapsed lungs - ●

Inflated lungs - ○

The mean shrinkage ± 1 SEM is shown for the collapsed lungs (mean density 0.8) and for lungs having a density of 0.25 to 0.4 (mean 0.32). The difference between the mean shrinkages shown is significant at $P = 0.001$.

increased, the % shrinkage increased up to $28\% \pm 3.39$ (SEM) for lungs inflated to 60 - 80% of total displaced lung volume.

The % shrinkage was inversely related to the density of the lungs at the moment of freezing (Fig XI), so that as the density decreased (i.e. as the lungs were inflated) the % shrinkage increased. No satisfactory regression equation could be found for this relationship; but the difference between the means at densities of 0.8 and 0.32 was statistically significant ($P = 0.001$).

The dependence of the % shrinkage on the degree of inflation of the lungs was investigated by the construction of a model. The mean collapsed volume measured for the lungs of guinea pigs was 5 cc. Suppose that various volumes of air are added to this mean cV, and assume that the shrinkage of the collapsed fraction is a constant 15%. The shrinkage of the added volumes of air can be estimated using the volume coefficient of thermal expansion of air ($0.00367/^{\circ}\text{C}$) applied over the range 30°C to -170°C , i.e. over 140°C . The volume V_L of these hypothetical lungs is therefore given by,

$$V_L = cV + V_{\text{air}} = 5 + V_{\text{air}}$$

and the shrinkage S_{\downarrow} (in cc) given by,

$$\begin{aligned} S_{\downarrow} &= 0.75 + 140.(0.00367 V_{\text{air}}) \\ &= 0.75 + 0.5 V_{\text{air}} \end{aligned}$$

When the calculated values of S_{\downarrow} were plotted against V_L the result was a straight line relationship, which very closely resembled that obtained from the measured values. This model is shown on Fig.IX as the broken line.

DISCUSSION

It is apparent that a constant shrinkage factor cannot be applied to inflated lungs following rapid freezing and subsequent freeze substitution fixation. The increased shrinkage seen in the inflated lungs would seem to relate to the volume of air within them, rather than to the absolute lung volume. A physical explanation of this finding is somewhat difficult when one considers that as the lungs are immersed in isopentane at -170°C a hard frozen shell forms within 0.5 seconds (Storey & Staub, 1962).

Two possible theories are proposed:

1. either the volume change occurs before the surface of the lungs is frozen, i.e. within 0.5 sec., or
2. as the lung freezes there are changes in the forces maintaining the degree of inflation, of sufficient magnitude, as to cause deformation of the already frozen tissues.

The first theory was tested on the lungs of one guinea pig following removal of the lungs by the method just described. A pressure-volume manoeuvre was performed on the lungs. They were then fully inflated and the airway closed. The airway pressure was continuously measured during immersion of the lungs in cooled isopentane.

No change in airway pressure was detected until 4 seconds after the lungs had been immersed in the isopentane. Thereafter, the airway pressure fell steadily. After 2 minutes, it had reached zero and after 10 minutes, it had reached an equilibrium of $-12\text{ cms H}_2\text{O}$.

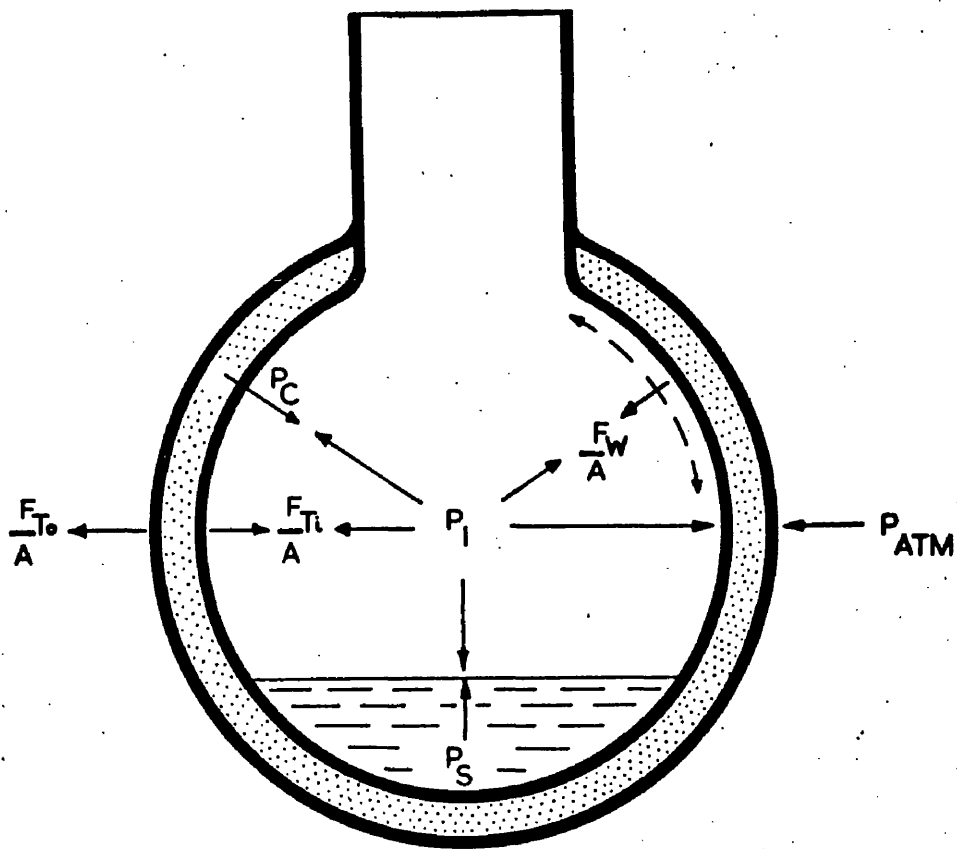
Since the lungs were held inflated with a constant inflation pressure, any change in the volume of the lungs would be reflected in a change in airway pressure. It would seem therefore, that there is little volume shrinkage during the first 4 seconds following rapid freezing of the inflated lung.

The balance of forces within the excised lung, when the airway is closed was considered in terms of a model (Fig. XII). It can be seen from this model that the pressure within the inflated lungs when the airway is closed, acts in an outward direction. To this is added those tissue elastic forces which also act in an outward direction. These two forces are balanced by five other forces acting in the opposite direction; inward acting tissue elastic forces, forces related to the internal curved surfaces (including surface tension), atmospheric pressure acting on the outside of the lungs, the pressure of gases in solution, and pulmonary capillary pressure.

The pressure of gases in solution was considered to influence this relationship for the following reasons: 1. the solubility of gases in solution is dependent on the temperature of the solution, 2. if the solution containing gases is suddenly frozen, the pressure equilibrium which existed at body temperature between the gaseous and liquid phases is upset such that the pressure of the gas in the gaseous phase is relatively greater than the gases trapped in ice.

The pulmonary capillary pressure was considered as a force which when the lung was inflated would tend to oppose inflation by increasing the stiffness of the lung, i.e. by decreasing lung compliance. However, in certain circumstances for example when pulmonary capillary pressure is high, it is possible that it will act in an outward direction. Rather in the way that the air pressure in a fully inflated rubber car tyre, maintains the shape of the tyre, by diminishing the forces acting towards the centre of the wheel. In this case the capillary pressure has a negative sign in equation (a).

As a mathematical model of the change in the volume of air within the excised inflated lung during rapid freezing of the lung, a formula (Equ. b) was derived based on equation (a). The exact manner by which the forces included in equation (a) will change during rapid freezing of the lung is not known; but



$$P_i + \frac{F_{To}}{A} = \frac{F_{Ti}}{A} + P_C + \frac{F_w}{A} + P_s + P_{ATM} \quad \text{Eq. (a)}$$

$$\text{TEMP. } \Delta V_i = \frac{\Delta RT}{\left[\frac{F_{Ti}}{A} - \frac{F_{To}}{A} + P_C + \frac{F_w}{A} \right] + \frac{V_s T}{KV_f} + P_{ATM}} \quad \text{Eq. (b)}$$

Fig. XII. The balance of forces within the excised inflated lung when the airway is closed.

Equ. (a) summarises these forces.

Equ. (b) relates the change in the volume of air with change in temperature to the change in the forces mentioned.

V_i air volume .

V_s volume of air in solution.

R gas constant .

K solubility coefficient .

V_f vol. of tissue fluid .

T absolute temperature .

P_i air pressure within the lung .

$\frac{F_{To}}{A}$ tissue forces per unit area acting outwards .

$\frac{F_{Ti}}{A}$ tissue forces per unit area acting inwards .

P_C pulmonary capillary pressure .

$\frac{F_w}{A} = p$ surface tension and the forces related to the curvature of the internal lung surfaces .

P_s the pressure of gases in solution .

P_{atm} atmospheric pressure .

some indication of this change can be got by incorporating the volume coefficient of thermal expansion of air and the solubility coefficient of air in water, into the equation.

It can be seen from the resulting relationship (equ. b) that the volume of air within an excised inflated lung with its airway closed will be reduced during rapid freezing of the lung, if any of the factors comprising the denominator on the right, is increased.

Applied correction factors:

The volume shrinkage correction factors which were subsequently applied to measurements on lungs which had been rapidly frozen and fixed by freeze substitution, were obtained from Tables relating percentage shrinkage to the degree of lung inflation. These tables were derived from the regression equation % shrinkage = $3.7 (\text{degree of inflation} - 15)^{0.35} + 13$, obtained from the measured values of shrinkage/degree of inflation (Fig. X). Degree of lung inflation was expressed as the volume of the lungs at the moment of freezing as a percentage of the total volume of the lungs.

VOLUME SHRINKAGE DUE TO WAX EMBEDDING, SECTIONING AND STAINING

Blocks were cut from lungs which had been rapidly frozen and fixed by the freeze substitution method. The block face was viewed under a dissecting microscope and the diameters were measured with a pair of vernier scale fine calipers.

The greatest diameter of the block face and the diameter at right angles on the same plane through its centre were measured.

The blocks were embedded in low melting point paraffin wax. When the wax had set the blocks were trimmed carefully with a scalpel blade until the block face was visible just under the surface of the wax. The diameters were again measured.

Sections were cut at 5μ , on a sliding microtome and run onto warm water. After each section had been cut the block was rotated by 90° so that the effects of compression by the microtome knife could be measured. Each section was picked up on a clean glass slide and the section diameters again measured viewed under a microscope. The sections were stained with haemalum and eosin and mounted in Canada Balsam. The diameters of the sections were finally measured on the stained and mounted preparations.

RESULTS

The mean linear shrinkages for the three stages of this part of the preparation of lung sections, are shown below. The area and volume shrinkages were derived from these values and are also shown.

TABLE VI - MEAN SHRINKAGE FACTORS

	A to B	B to C	C to D	A to D
$\bar{m}f_L = \frac{A}{B}$ $\pm 1 \text{ SEM}$	1.003 ± 0.001 (0.37%)	1.034 ± 0.022 (3.2%)	1.008 ± 0.01 (0.81%)	1.045 (4.38%)
$\bar{m}f_A = f_L^2$ $\pm 1 \text{ SEM}$	1.006 ± 0.001 (0.59%)	1.07 ± 0.05 (6.37%)	0.016 ± 0.01 (1.59%)	1.093 (8.61%)
$\bar{m}f_V = f_L^3$ $\pm 1 \text{ SEM}$	1.009 ± 0.001 (0.98%)	1.108 ± 0.08 (9.32%)	1.025 ± 0.02 (2.38%)	1.144 (12.67%)

A = the fixed block of lung.

B = the embedded block.

C = the section cut at 5μ .

D = the stained and mounted section.

Percentage shrinkages are given in the brackets.

The factors f_L , f_A and f_V are the linear, area and volumetric factors which when multiplied by the processed dimensions will give the original dimensions. Thus for example, a diameter d_2 measured on a stained and mounted section of lung represents the diameter d_1 which would have existed in a block of fixed lung, and is obtained from

$$d_1 = 1.045d_2$$

Summary of correction factors for shrinkage due to processing:

The shrinkage factor which was applied in any individual case was the multiple of the factor for shrinkage due to rapid freezing and fixation by freeze substitution, and the factor for shrinkage due to wax embedding, sectioning and staining. The factors relating to shrinkage due to rapid freezing and fixation, were obtained from a curve relating percentage shrinkage and degree of inflation. The factors for shrinkage due to embedding, sectioning and staining, were obtained from the preceding table.

It was thus possible to obtain measurements of dimensions related to those which would have existed in the fresh lung just before rapid freezing.

PERFUSION FIXATION FOR ELECTRON MICROSCOPY

Material and methods:

Two guinea pigs were anaesthetized with intraperitoneal pentobarbitone sodium (60mg/kg). A tracheostomy was performed and the trachea cannulated, The sternum was split and the anterior rib cage dissected out by cutting through the ribs in the mid axillary line. The inferior vena cava, and the aorta were cannulated. The lungs were then fully inflated with air from a syringe and the airway clamped.

Perfusion via the caval cannula, with normal saline at 37°C and a perfusion pressure of 5 cm H₂O, was begun. After 5 minutes the perfusate was switched to a 4% solution of gluteraldehyde in 0.1 molar potassium phosphate buffer at pH 7.4. In one of the animals the lungs were held inflated during perfusion with fixative, while in the other animal the lungs were allowed to collapse slowly after stopping the saline perfusion. When they had collapsed completely the perfusion with gluteraldehyde was begun. The perfusion pressure during fixation of the inflated lungs was 5 cm H₂O, whereas in the collapsed lungs the perfusion pressure necessary to maintain adequate perfusion was 20 - 26 cms H₂O. Perfusion was continued for 10 minutes.

Preparation of blocks:

When the lungs were fixed, they were dissected out. The lower right and lower left lobes of the lungs of both animals were cut free and block of 0.5 cm³ were gently cut with a sharp razor blade. These were immersed in 4% buffered gluteraldehyde, trimmed and sliced each slice being about 0.5mm thick.

The lung slices were then carefully cut into small blocks, 0.5 cm³ under a drop of gluteraldehyde. A total of 6 blocks per lung lobe were obtained. They were transferred to a 3% solution of sucrose buffered with potassium phosphate

to pH 7.4, and stored overnight at 5°C.

The blocks were then post fixed in a 1% solution of OsO₄ buffered with potassium phosphate to pH 7.4, for 30 minutes. They were then taken up through a series of graded alcohols to propylene oxide and infiltrated with Araldite (CIBA). The infiltrated blocks were then transferred to capsules and embedded in Araldite.

500 Å thick sections were cut on an ultramicrotome (L.K.B. Ultratome), mounted on copper grids, and stained with uranyl acetate and lead citrate. The stained sections were viewed with an electron microscope (AEI 6B Electron Microscope).

PRACTICAL PROCEDURES USED FOR MORPHOMETRIC
ANALYSIS

MEASUREMENT OF THE VOLUME OF LUNG
PARENCHYMA (RESPIRATORY TISSUE)

The entire lung was sectioned vertically in 5μ slices. Every hundredth section was retained. The 140 resulting sections were stained with haemalum and eosin and mounted in Canada Balsam. The slides were placed in a photographic enlarger and $8 \times 10''$ enlargements made of the whole lung section.

Tracing paper was placed over the enlargement and the section outline traced. The outlines of all airways except respiratory bronchioles and alveolar ducts, and all visible blood vessels were traced. The traced contour of the lung section was then cut out and the paper weighed (W_T). The contours of the major airways and blood vessels were then cut out, and the collected pieces weighed (W_a). The remaining tracing paper which represented the area of the lung parenchyma on the section, was also weighed (W_b) as a check against loss during cutting. Thus $W_T = W_a + W_b$.

In this way an accurate measure was obtained of the relative area occupied by the lung parenchyma on random sections of whole lungs. This was expressed as a percentage of the total section area,

$$\text{area fraction of lung parenchyma, } \psi = \frac{W_b \cdot 100}{W_T}$$

This has been shown to be equal to the volume fraction (page 67). Knowing the total volume of the lungs (TV_L), the total volume of the parenchymal portion is given by $\psi \cdot TV_L$.

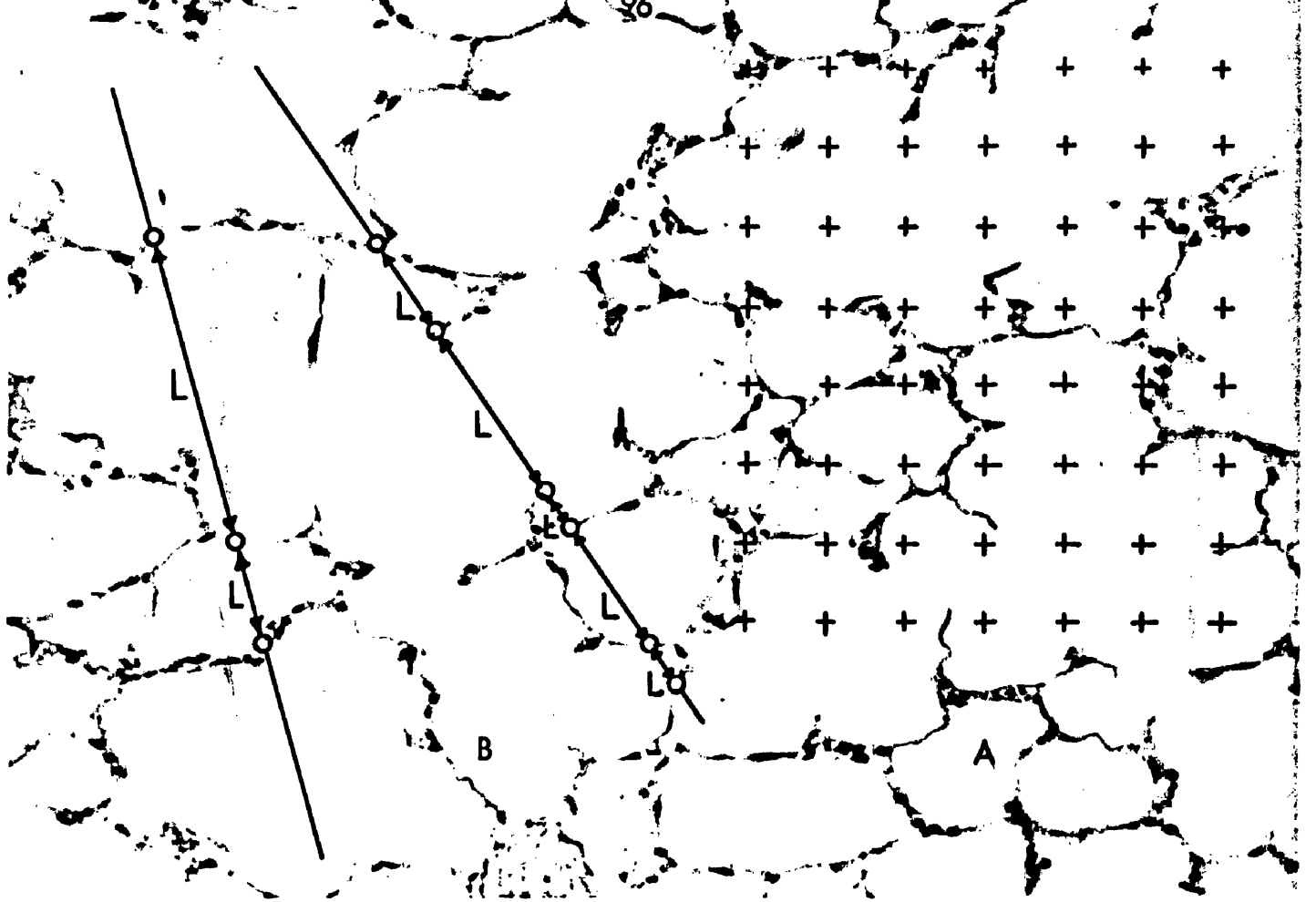


Fig. XIII. A. Measurement of volumes by point counting.
B. Measurement of surface area by linear intercepts.

MEASUREMENT OF VOLUMES BY POINT COUNTING (Fig. XIII A)

Photographic enlargements (8" x 10") were obtained of randomly selected fields by the method described on page 83 . A grid of 1000 regularly spaced points was superimposed over the photograph and the number of points lying on alveoli (P_a), alveolar ducts (P_d) and alveolar tissue (P_t) were counted. Each of these was expressed as a fraction of the total number of points. Using Equ. 5, the area and hence the volume fractions of each component were obtained thus,

$$\frac{P_a}{1000} = \underline{V}_a, \quad \frac{P_d}{1000} = \underline{V}_d, \quad \frac{P_t}{1000} = \underline{V}_t$$

The total volume of each component was obtained by multiplying the mean volume fraction by the total volume of the parenchymal portion of the lung ($\Psi \cdot TV_L$).

Therefore the total alveolar volume $TV_a = \underline{V}_a \cdot \Psi \cdot TV_L$ and so on. Each of the volumes thus obtained was then corrected for shrinkage due to tissue processing.

MEASUREMENT OF SURFACE AREAS BY MEAN CHORD LENGTHS (Fig. XIII)

A grid containing 5 randomly orientated straight lines of equal length was superimposed on photographic enlargements of lung sections. The number of intersections (n_a) which each line made with the alveolar surface was counted. Knowing the magnification of the enlargement and the length of the line, the actual length measured on the section was calculated.

The mean chord length (L_m) is the mean interval between each intersection and is given by $\frac{n \cdot L}{\sum n_a} = L_m$,

where: n is the number of estimations,

L is the total length of the line, and

$\sum n_a$ is the sum of alveolar surface intersections.

The total surface area of alveoli (TSa) was obtained from Equ. 8,

$$TSa = \frac{4 \cdot \Psi \cdot TV_L}{L_m}$$

where: $\Psi \cdot TV_L$ is the volume of the respiratory portion of the lung.

The surface area was also estimated using a formula derived by Weibel (1963a). Total alveolar surface area (Sat) is given by,

$$TSa = TNa^{\frac{1}{3}} \cdot \delta_a (\underline{V}_a \cdot \Psi \cdot V_L)^{\frac{2}{3}} \quad (\text{Equ. 14})$$

where: Nat is the total number of alveoli, measured by direct counting,

δ_a is the surface to volume coefficient (for calculation see below),

\underline{V}_a is the alveolar volume fraction, measured by point counting, and $\Psi \cdot TV_L$ is the volume of the lung parenchyma.

SURFACE TO VOLUME RATIOS

The surface to volume ratios (δ_a) of alveoli were obtained from Equ. 9 in a modified form (Weibel, 1963), so that,

$$\delta_a = \frac{4(\varphi \cdot TV_L)^{\frac{1}{3}}}{TN_a^{\frac{1}{3}} \cdot V_a^{\frac{2}{3}} \cdot L_m} \quad (\text{Equ. 15})$$

Each of the factors on the right were determined independently:

TNa the total number of alveoli,

V_a the volume fraction of alveoli,

L_m the mean chord length,

$\varphi \cdot TV_L$ the volume of the parenchymal portion of the lung.

TOTAL NUMBER OF ALVEOLI

The total number of alveoli (TNa) was measured by direct counting. A grid enclosing 16.000 mm^2 was superimposed over photographic enlargements of lung sections. Knowing the magnification which in most cases was $\times 180$, the area of lung section which the grid enclosed was calculated (i.e. at $\times 180 = 0.4984 \text{ mm}^2$). The number of alveoli within this area were counted. Alveoli which bordered on the upper and left margins of the grid were included whereas those bordering on the lower and right margins were ignored. The number of alveoli per cm^2 was then calculated and from this the number per cm^3 obtained.

Using Equ. 10 the total number of alveoli (TNa) was calculated, thus,

$$TNa = \frac{(n/\text{cm}^3) \cdot \varphi \cdot TV_L}{\beta \cdot V_a^{\frac{1}{2}}} \cdot K \quad (\text{Equ. 16})$$

where: β is a coefficient relating the mean cross sectional area of the alveolus to its mean volume, the value of 1.55 was used as suggested by Weibel & Gomez (1962),

K is a distribution coefficient (see page 70) and was given an arbitrary

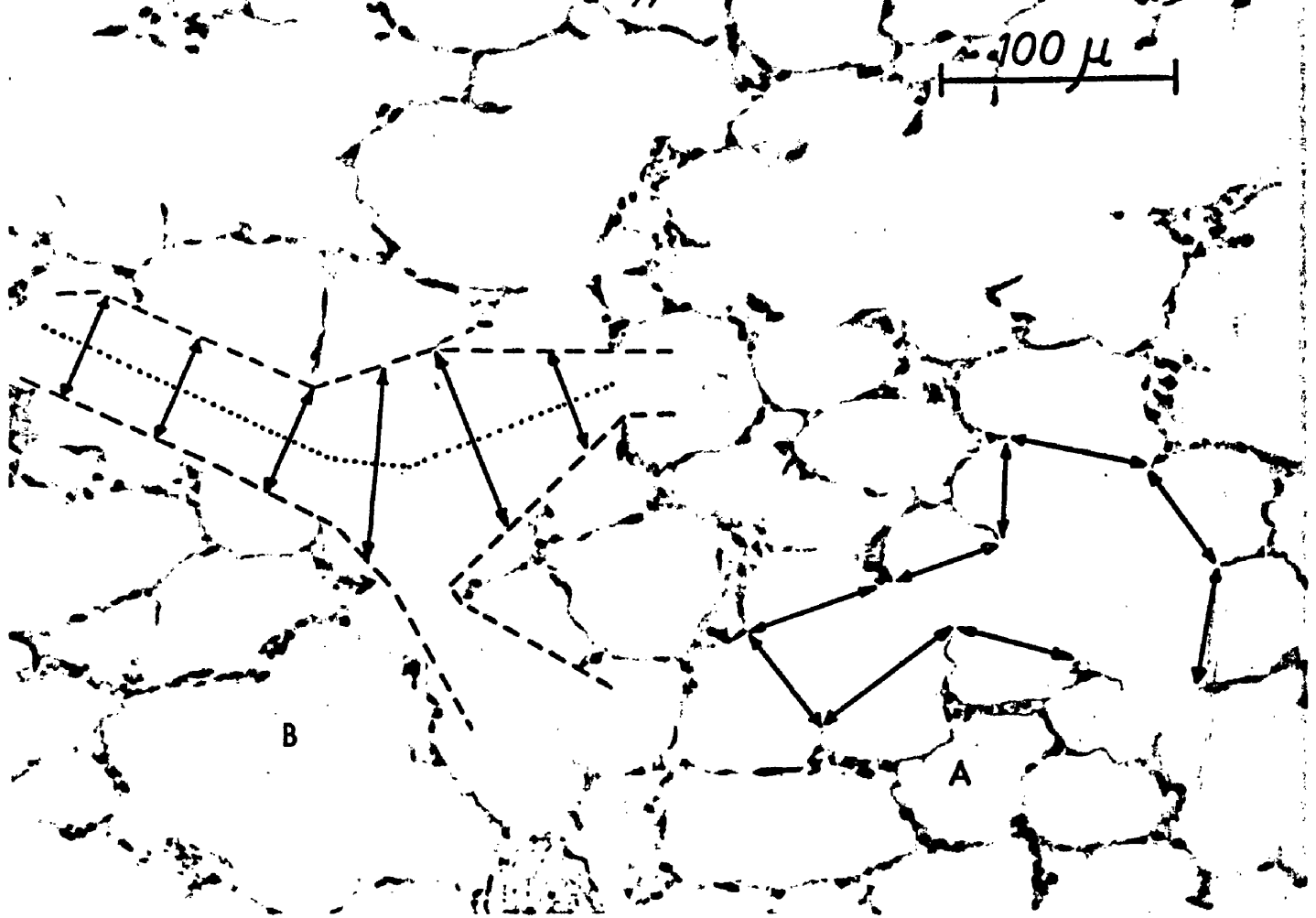


Fig. XIV. A. Measurement of alveolar mouth diameter.
 B. Measurement of alveolar duct diameter.

value of 1.05,

$\frac{V}{V_a}$ is the volume fraction of alveoli, and
 $\Psi.TV_L$ is the volume of the parenchymal portion of the lung.

MEASUREMENT OF LINEAR DIMENSIONS (Fig. XIV)

The diameters of alveolar mouths (Fig. XIV A) and the diameters of alveolar ducts were measured directly on photographs of lung sections. The alveolar duct diameters were measured at regular intervals along a line drawn centrally along the length of the duct and were measured at right angles to this line. (Fig. XIV B).

All diameters were corrected for frequency distribution by the application of Equ. 11. Thus the true diameters (d) were given by,

$$d = \frac{4 d_x}{11}$$

where d_x was the measured diameter. The values for d were then corrected for shrinkage due to processing.

RESULTS OF MORPHOMETRIC ANALYSIS

The lung volumes, body weights, and periods of ventilation are given for each animal in Table VII.

VOLUMETRIC COMPOSITION

Fractional volume of the lung parenchyma:

The volume fraction occupied by the respiratory portion of the lungs was estimated 543 times. The mean volume fraction (± 1 SD) was 0.9165 ± 0.0053 . For subsequent calculations a value of 0.9 was used. The loss of accuracy by doing this was insignificant. The value found in this study agrees well with other estimates. Weibel (1963) gives a value of 0.905 ± 0.038 for the fractional volume of lung parenchyma.

FRACTIONAL ALVEOLAR VOLUMES

The alveolar, alveolar duct and respiratory tissue area fractions, (volume fractions) are given in Table VIII. Fig. XV shows the alveolar volume fractions as a function of the percentage total volume of the lungs ($\%TV_L$) for each of the four ventilation groups. The standard deviations and the means for each animal are shown.

In the upper control curve it can be seen that as lung inflation increases the alveolar volume fraction rises from 0.52 at 28% TV_L to 0.62 at 45% TV_L and thereafter falls back to 0.51 at 60% TV_L .

The mean alveolar volume fraction was significantly higher in the mid-inflation range (35- 55% TV_L) than in low and high inflations (30% TV_L & 60% TV_L), ($P < 0.001$).

No difference was found between alveolar duct volume fractions from lungs at different degrees of inflation.

Hyperventilation reduced the alveolar volume fraction, so that after

LUNG VOLUMES

Animal	Weight gms.	cV cc.	TV _L cc.	TV _{air} cc.	V _{Lf} cc.	%TV _L	QV _{Lf} cc.	Vent. time mins.
63/68:1	486.2	4.57	31.84	15.33	19.90	62.50	17.93	5
63/68:2	482.1	4.50	31.50	1.00	5.50	17.46	4.95	0
63/68:3	442.5	4.20	29.00	4.05	8.25	28.45	7.43	0
63/68:4	640.2	5.95	41.99	0.39	6.34	15.10	5.72	10
68/68:1	420.0	3.98	27.57	2.83	6.81	24.70	6.14	10
68/68:2	447.7	4.23	29.30	13.77	18.00	61.43	16.20	0
69/68:1	358.2	3.42	23.40	7.14	10.56	45.13	9.52	0
69/68:2	360.0	3.42	23.46	7.42	10.84	46.20	9.77	10
70/68:1	417.5	3.95	27.40	8.55	12.50	45.62	11.25	0
70/68:2	372.9	3.55	24.30	5.70	9.25	38.07	8.23	0
70/68:3	472.0	4.45	31.34	18.90	23.35	74.50	21.04	10
59/68:1	581.5	5.40	38.00	00.00	5.40	14.21	4.86	5
59/68:2	545.0	5.10	35.68	2.18	7.28	20.40	6.56	15
77/68:1	435.0	4.10	28.49	8.75	12.85	45.10	11.58	15
77/68:2	418.9	3.95	27.40	3.00	6.95	25.36	6.26	2
61/68:1	462.5	4.35	31.00	18.86	23.21	75.70	21.11	1
61/68:2	737.8	6.85	48.00	27.50	34.35	71.56	30.92	0
61/68:3	430.5	4.10	28.50	12.05	16.15	56.66	14.54	0
61/68:4	470.0	4.42	30.70	16.98	21.40	69.70	19.28	15
11/69:1	510.0	4.80	33.50	19.45	24.25	72.40	21.83	5
11/69:2	667.8	6.20	43.50	18.50	24.70	48.28	22.23	5
11/69:3	523.5	5.00	35.00	25.50	30.50	87.14	31.41	15
11/69:4	428.0	4.03	28.02	9.56	13.59	48.50	12.25	5

TABLE VII

cV collapsed lung volume

TV total volume of lung

TV_{air} total volume of tidal airV_{Lf} volume of lung at moment of freezingQV_{Lf} volume of lung parenchyma at moment of freezing

AREA FRACTIONS: CONTROL LUNGS

Animal	Alveoli ± 1 S.E.M.	Alveolar Ducts ± 1 S.E.M.	Tissue and Vessels ± 1 S.E.M.
63/68:2	0.5260 \pm 0.023	0.2180 \pm 0.035	0.2552 \pm 0.014
63/68:3	0.5310 \pm 0.035	0.2200 \pm 0.045	0.2387 \pm 0.010
68/68:2	0.5250 \pm 0.048	0.1750 \pm 0.033	0.2875 \pm 0.017
69/68:1	0.6350 \pm 0.016	0.2280 \pm 0.015	0.1370 \pm 0.005
70/68:1	0.6240 \pm 0.017	0.1483 \pm 0.039	0.2281 \pm 0.021
70/68:2	0.6070 \pm 0.009	0.2160 \pm 0.008	0.1790 \pm 0.004
61/68:2	0.5280 \pm 0.014	0.2510 \pm 0.035	0.2249 \pm 0.024
61/68:3	0.5520 \pm 0.010	0.2630 \pm 0.021	0.1854 \pm 0.001

Hyperventilated for 5 mins.

HYPERVENTILATED LUNGS

63/68:1	0.4030 \pm 0.012	0.2913 \pm 0.007	0.3057 \pm 0.01
11/69:1	0.4210 \pm 0.001	0.2695 \pm 0.014	0.3091 \pm 0.01
11/69:2	0.4570 \pm 0.017	0.2972 \pm 0.024	0.2452 \pm 0.01
11/69:4	0.4162 \pm 0.004	0.2550 \pm 0.006	0.3288 \pm 0.009

Hyperventilated for 10 mins.

68/68:1	0.3483 \pm 0.008	0.1986 \pm 0.008	0.4531 \pm 0.006
63/68:4	0.3197 \pm 0.010	0.1654 \pm 0.015	0.5149 \pm 0.010
69/68:2	0.3901 \pm 0.008	0.2752 \pm 0.010	0.3347 \pm 0.010
70/68:3	0.3993 \pm 0.010	0.3105 \pm 0.010	0.2902 \pm 0.010

Hyperventilated for 15 mins.

59/68:2	0.3172 \pm 0.014	0.2064 \pm 0.009	0.4764 \pm 0.005
77/68:1	0.3316 \pm 0.010	0.2901 \pm 0.014	0.3783 \pm 0.008
61/68:4	0.3521 \pm 0.004	0.3223 \pm 0.007	0.3256 \pm 0.004
11/69:3	0.3460 \pm 0.020	0.3390 \pm 0.024	0.3122 \pm 0.017

TABLE VIII

FRACTIONAL COEFFICIENT

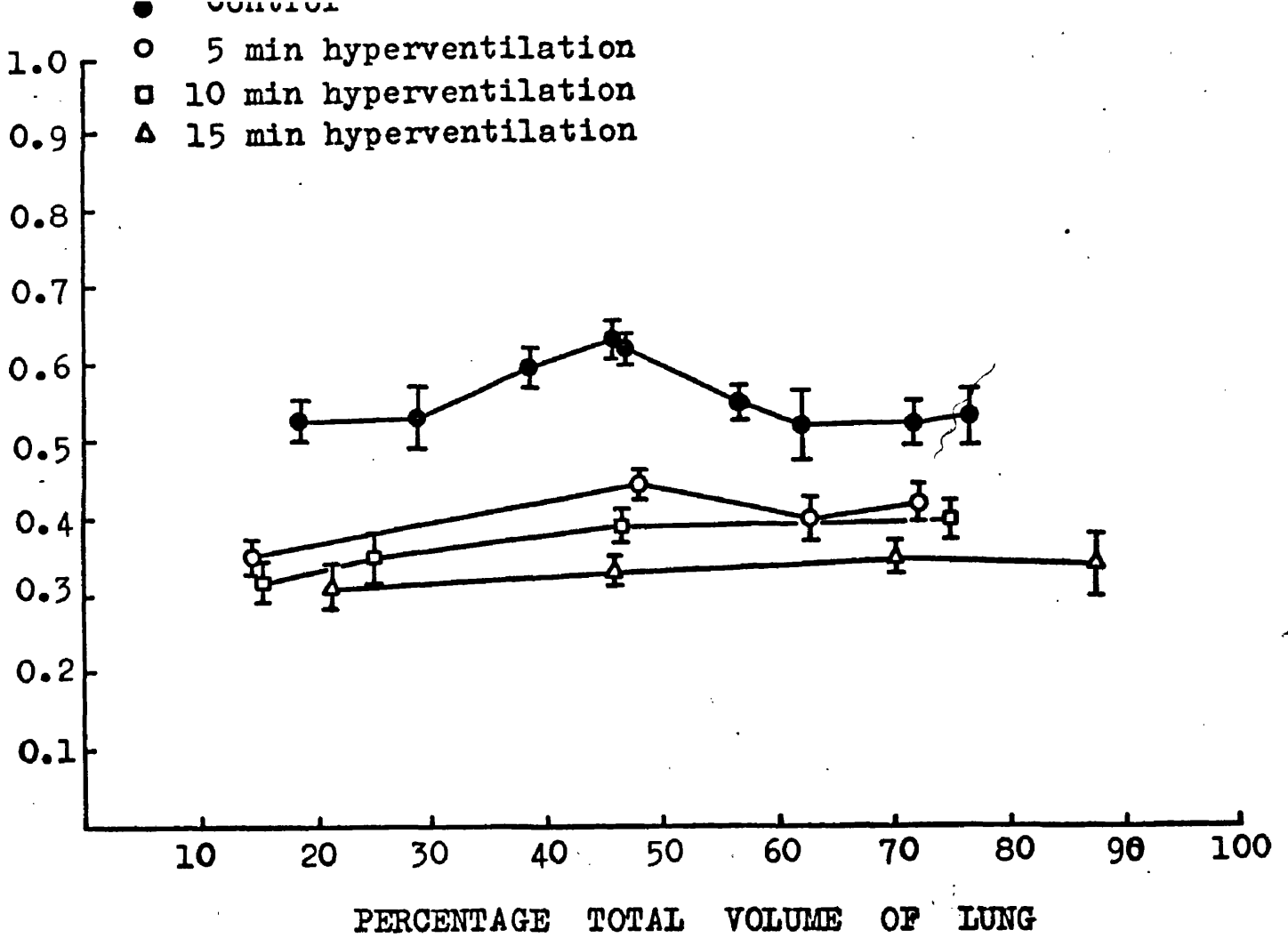


Fig. XV

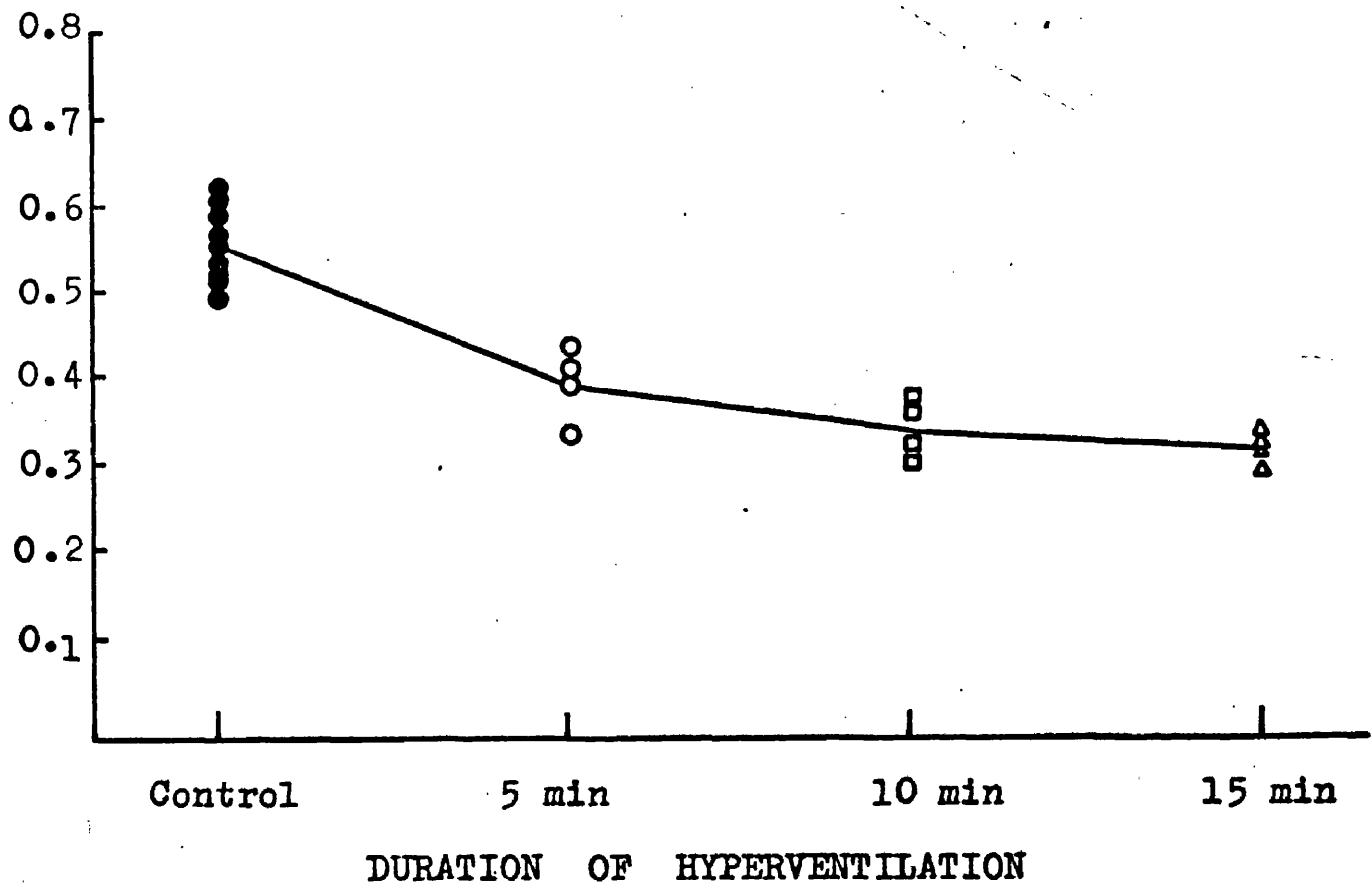


Fig. XVI

15 minutes hyperventilation it was 0.3. There was no difference here between the mean alveolar volume fractions at the four degrees of inflation.

Fig. XVI shows the alveolar volume fraction plotted against the duration of hyperventilation. The difference between the means for the control and the 5 minute hyperventilation group was highly significant at $P' < 0.001$. The difference between the means for the 5 minute and 10 minute hyperventilation groups was smaller but still significant ($P' = 0.05$). The means after 10 minutes and 15 minutes hyperventilation were similar.

However analysis of the alveolar volume fractions of lungs of comparable degree of inflation, showed that there was a significant difference between these two groups when the lungs were inflated to more than 40 % TV_L (see Fig. XV).

Hyperventilation did not alter the alveolar duct volume fraction.

TOTAL ALVEOLAR AND ALVEOLAR DUCT VOLUMES

The total alveolar and alveolar duct volumes are given in Table IX.

Fig. XVII shows the total alveolar volume (TV_a) plotted against % TV_L , for each of the four ventilation groups.

It can be seen for all lungs that as % lung inflation increases, the total alveolar volume increases. The upper control curve shows that the total alveolar volume reaches a plateau at 80 % TV_L beyond which little change in the total alveolar volume occurs.

Regression analysis was carried out in the data from the control lungs, and it was found that the correlation followed a simple parabolic function ($TV_a = (0.15 \%TV_L)^2 - 0.25 \%TV_L + 1.35$) significant at $P' < 0.001$. However a straight line correlation could also be fitted to this data ($TV_a = 0.1997 \%TV_L - 2.045$); but was of slightly less significance ($P' = 0.01$). The curves shown for the hyperventilated groups are best fitted curves drawn by eye.

Hyperventilation shifted the curve down and to the right, so that after

TABLE IX - TOTAL ALVEOLAR VOLUMES AND TOTAL ALVEOLAR DUCT VOLUMES

$\% TV_L$	TV_a cc	TV_d cc	Vent. time min.
17.46	2.60	1.09	0
28.45	3.94	1.71	0
38.07	4.99	1.81	0
45.13	6.04	2.19	0
45.62	7.01	1.69	0
56.66	8.02	3.82	0
61.43	8.50	2.92	0
71.56	11.32	7.73	0
75.7	12.03	5.74	1
14.21	1.72	0.86	5
48.5	5.09	2.86	5
62.5	7.22	4.39	5
72.4	9.19	5.89	5
15.1	1.82	1.11	10
24.7	2.13	1.28	10
46.2	3.81	2.73	10
74.5	8.40	6.12	10
20.4	2.08	1.42	15
45.1	3.83	2.85	15
69.7	6.78	5.91	15
87.14	10.86	10.67	15

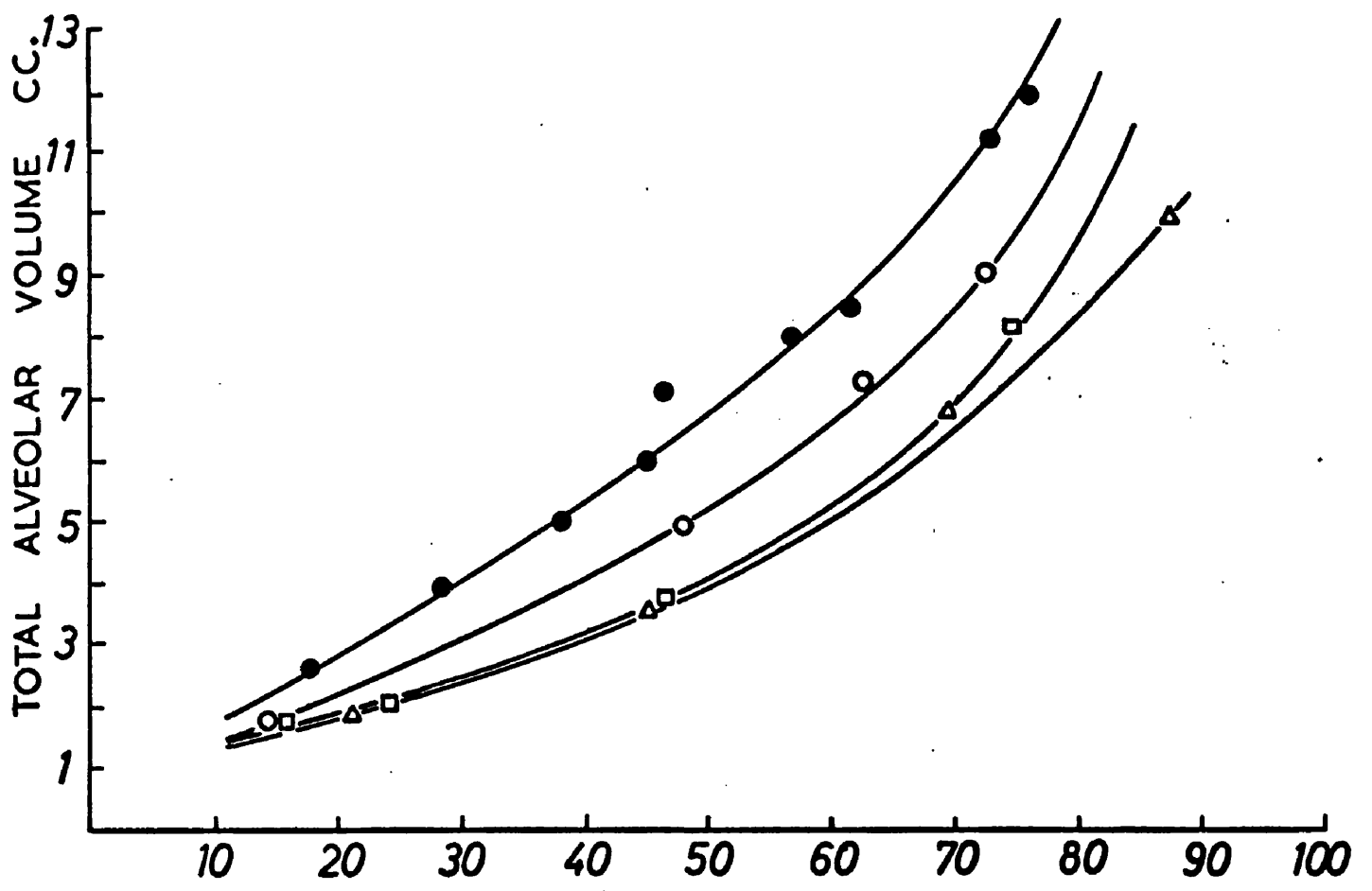


Fig. XVII

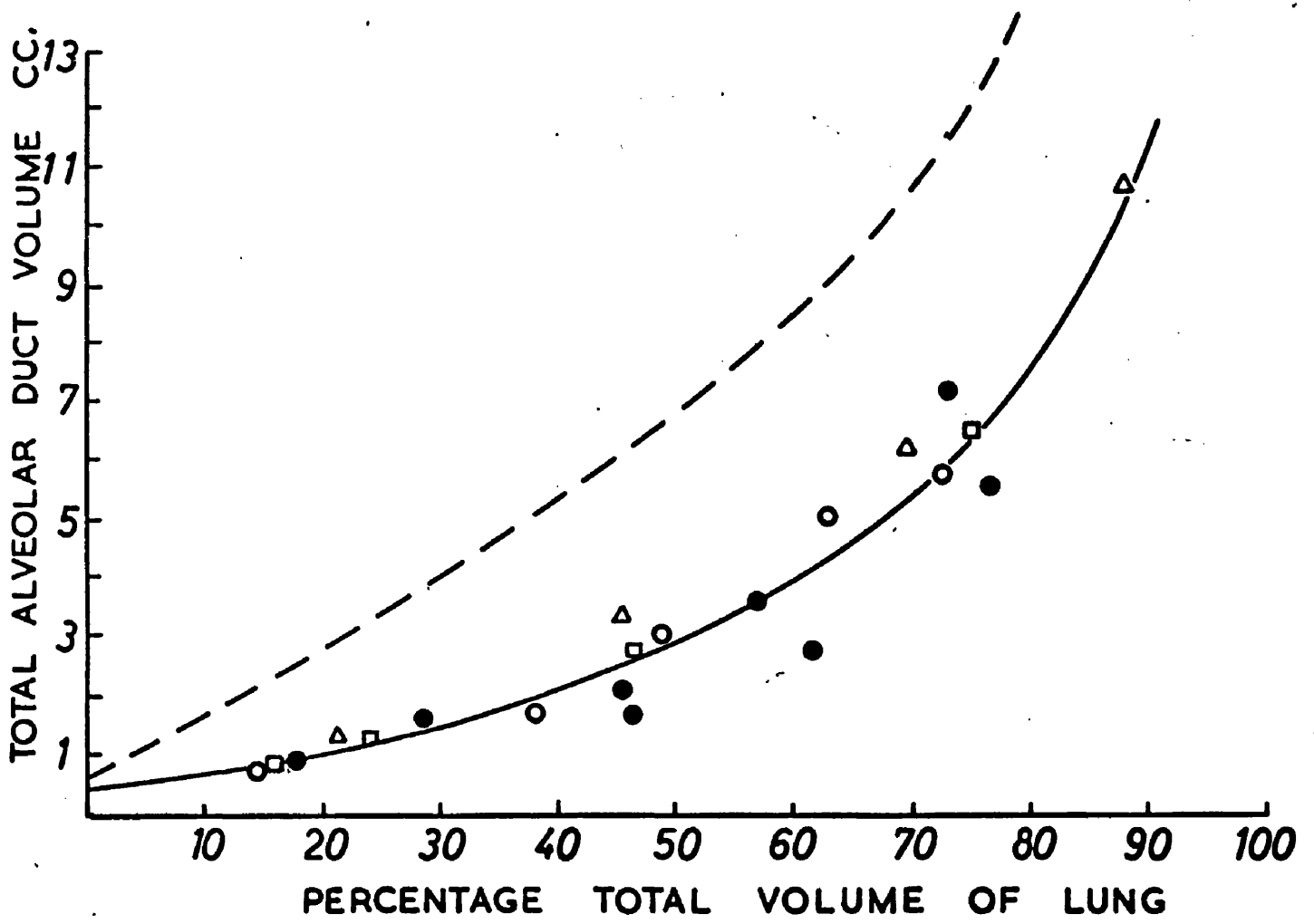


Fig. XVIII

15 minutes hyperventilation, the total alveolar volume at 50 % TV_L was 40% less than the control values. Further increase in lung volume did not change the absolute difference between the controls and the 15 minute hyperventilation group; but the fractional difference increased. Thus at 80% TV_L the TV_a was 30% less in the 15 minute hyperventilated group than in the controls.

Fig. XVIII shows the total alveolar duct volume (TV_d) plotted against % TV_L . The broken line is the curve shown in Fig. XVII for the total alveolar volume in the control lungs, and is inserted in Fig. XVIII so that comparison can be made between the change in TV_a and the change in TV_d with lung inflation.

No statistical difference was found between the values for TV_d from the four ventilation groups. Regression analysis was done on the combined data from all groups and was found to follow the function,

$$TV_d = 0.23 \%TV_L^{0.64} + 0.5 \quad (P' = 0.05).$$

It can be seen that there is relatively little change in TV_d until the lung is inflated to more than 40 % TV_L . Thereafter the TV_d rises steeply and is maximal about 90 % TV_L .

Fig. XIX shows the values for TV_a plotted against the corresponding values for TV_d . The curves shown are best fitted curves, drawn by eye.

The data corresponding to lungs of low % TV_L are to the left, and of high % TV_L to the right.

It can be seen that there is a proportionately greater increase in the total alveolar volume initially. The range in which TV_a and TV_d increase to the same extent corresponds to above about 40 % TV_L .

Hyperventilation resulted in a straight line relationship between TV_a and TV_d , in contrast to the control curve (Fig. XIX). TV_a decreased progressively with the duration of hyperventilation; but TV_d was unaltered after hyperventilation. The reduction in the ratio of TV_a/TV_d is indicated by a change in the slope of

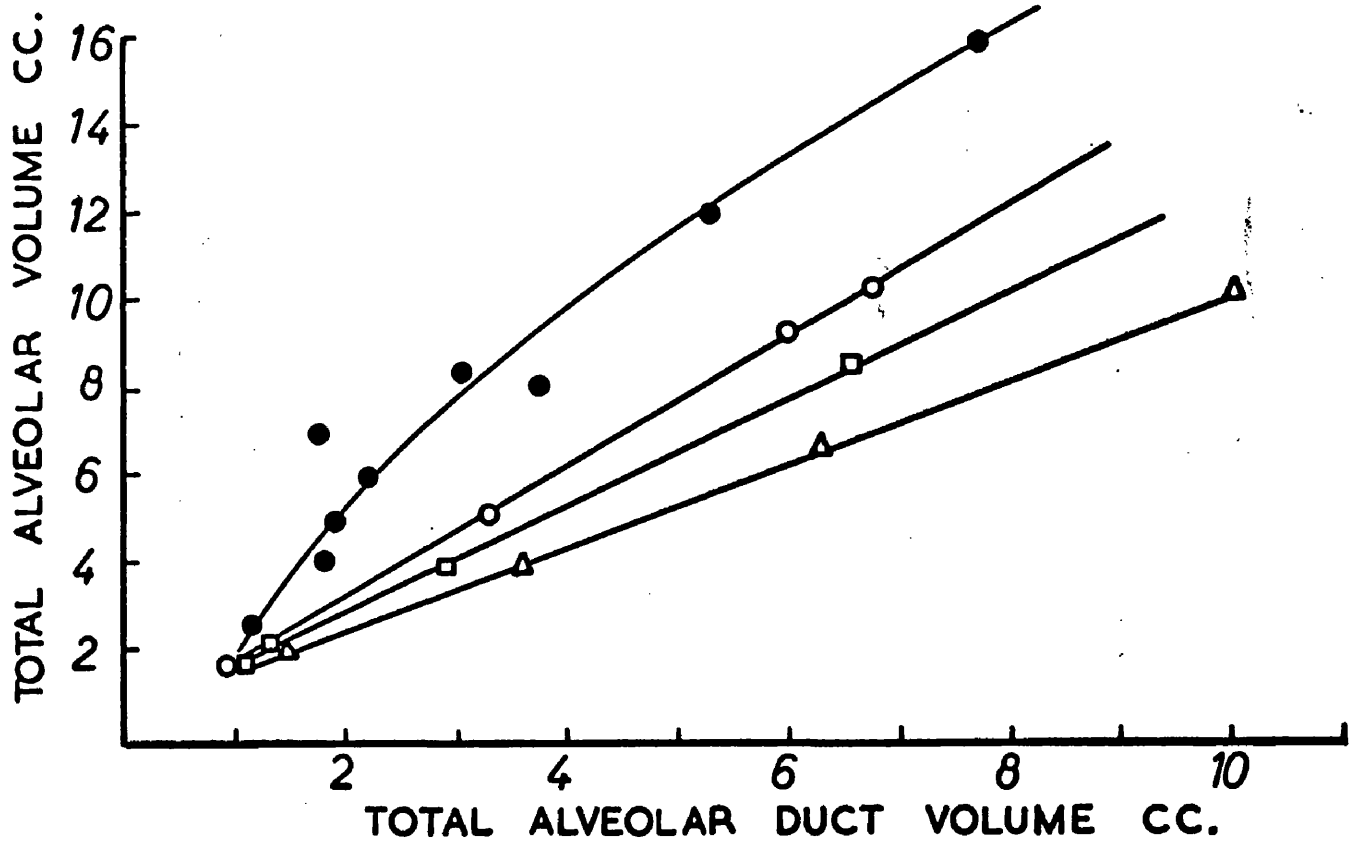


Fig. XIX

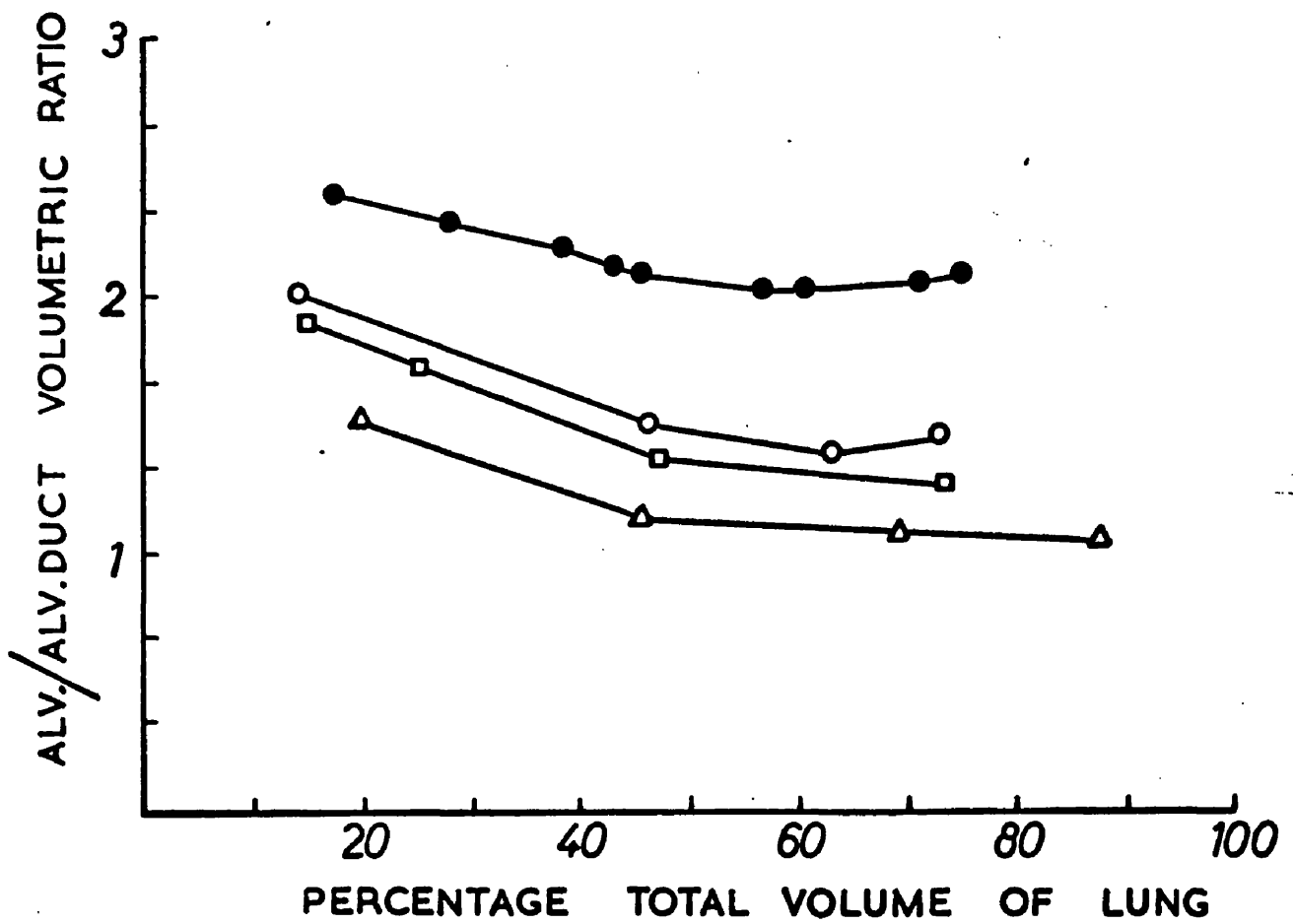


Fig. XX

the curves. Fig. XX shows the ratio of TV_a/TV_d as a function of % TV_L . In the control lungs the TV_a/TV_d ratio fell from 2.4 at 20 % TV_L to 2.0 at 60% TV_L and thereafter levelled off. This would indicate that at low lung volumes the TV_a is higher compared with TV_d but that above 40 % TV_L , the extent to which TV_a and TV_d increase is equal.

Hyperventilation reduced the TV_a/TV_d ratio; but the overall shape of the curves remained the same. After 15 minutes hyperventilation the TV_a/TV_d ratio had fallen to 1.5 at 20 % TV_L and levelled off at 1.1 at 45 % TV_L .

TOTAL NUMBER OF ALVEOLI

The total number of alveoli (TN_a) was calculated using Eq. 16 and are given in Table X for all animals studied. Fig. XXI shows the values of TN_a plotted against body weight. The relationship was found to follow a linear correlation, given by $TN_a = 10^6(0.0922 \text{ B.Wt.} + 10.47)$. The correlation coefficient was 0.8218 ($P' = 0.001$).

Because of a possible high influence on the correlation by the four points on the extreme right, regression analysis was repeated excluding these values.

The result was a linear regression which followed the relation, $TN_a = 10^6(0.103 \text{ B.Wt.} + 33.7)$, correlation coefficient 0.6836 ($P' = 0.001$).

Since these two basic correlations were not changed by excluding those values which were considered to have an undue influence on the correlation, it was decided that in future calculations requiring a measure of TN_a , the individual values would be used. It was apparent that a mean value for TN_a would not be applicable in calculations where the result depended to a large extent on the value of TN_a , for example, in the calculation of the shape coefficients.

ALVEOLAR SURFACE AREA

The total alveolar surface area (TS_a) as measured by mean chord lengths

TOTAL NUMBER OF ALVEOLI

Animal	QV_{Ls}	V_{-a}	$TN_a \times 10^{-6} \pm 1 \text{ S.E.M.}$
63/68:1	13.26	0.4030	49.4 \pm 1.178
63/68:2	4.25	0.5260	56.2 \pm 2.099
63/68:3	5.78	0.5310	50.5 \pm 1.213
63/68:4	4.86	0.3197	69.6 \pm 1.301
68/68:1	4.85	0.3483	42.3 \pm 1.155
68/68:2	12.12	0.5250	57.1 \pm 1.509
69/68:1	7.22	0.6350	42.3 \pm 2.167
69/68:2	7.32	0.3901	48.7 \pm 1.668
70/68:1	8.53	0.6240	47.2 \pm 1.925
70/68:2	6.39	0.6070	38.3 \pm 1.406
70/68:3	15.56	0.3993	55.8 \pm 1.837
59/68:1	4.17	0.3530	64.3 \pm 1.326
59/68:2	5.24	0.3172	51.7 \pm 1.401
77/68:1	8.68	0.3316	52.4 \pm 1.789
77/68:2	5.46	0.3770	58.7 \pm 1.261
61/68:1	15.80	0.5650	43.6 \pm 1.252
61/68:2	23.13	0.5280	77.2 \pm 1.197
61/68:3	10.88	0.5520	61.1 \pm 1.266
61/68:4	14.26	0.3521	52.2 \pm 1.332
11/69:1	16.33	0.4210	59.1 \pm 2.280
11/69:2	16.86	0.4570	81.3 \pm 2.254
11/69:3	20.53	0.3460	52.8 \pm 2.021
11/69:4	9.18	0.4162	53.4 \pm 1.861

TABLE X

QV_{Ls} Volume of lung parenchyma of fixed lung

V_{-a} Fractional alveolar volume

TN_a Total number of alveoli

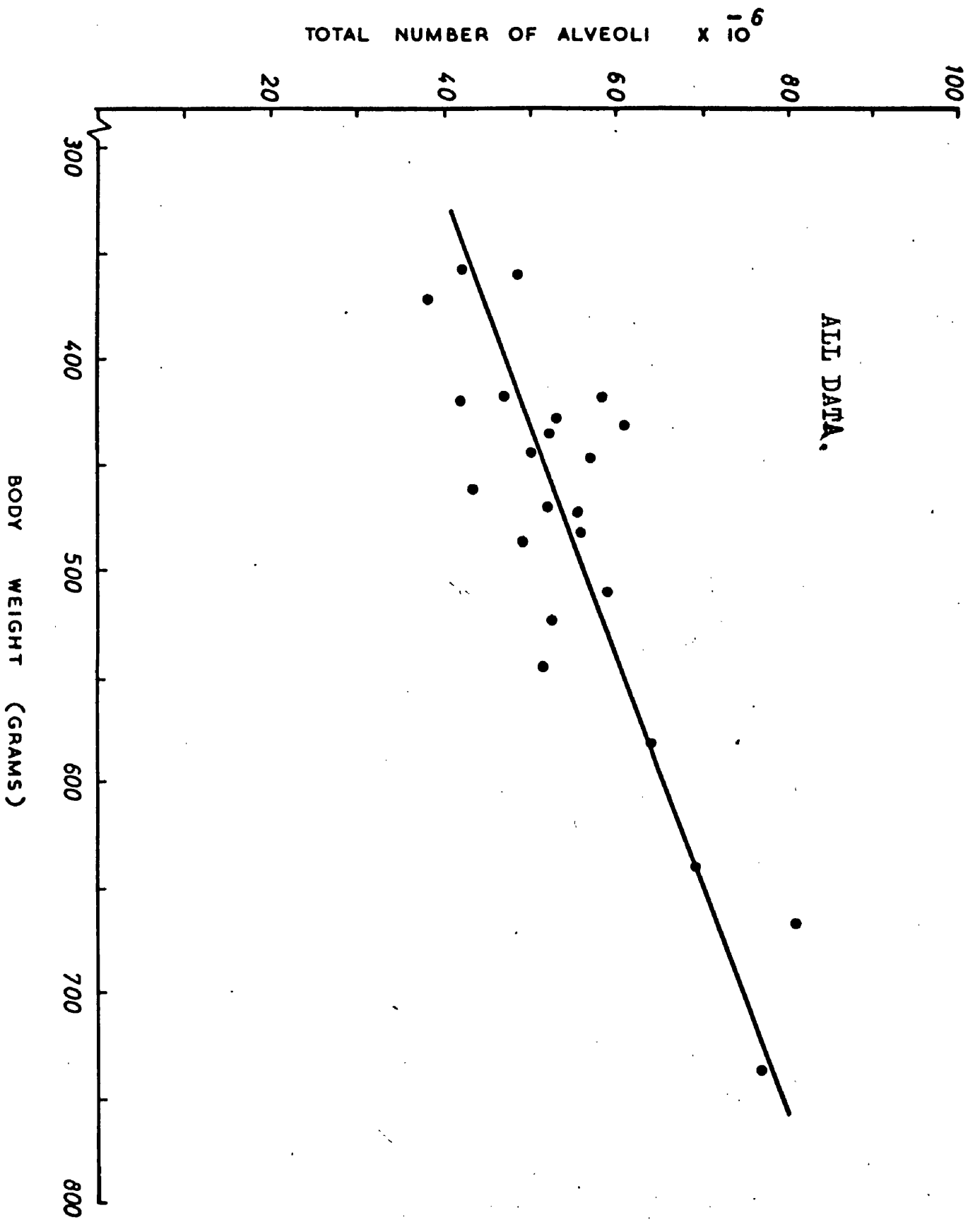


FIG. XXI

is given in Table XI for each animal studied. Regression analysis was performed on the values of total alveolar surface area against % TV_L; but no satisfactory correlation could be found. In view of the relationship between body weight and TN_a it appeared that body weight might influence the values for TS_a. A formula was therefore derived, based on Equ. 14,

$$TS_a = TN_a^{\frac{1}{3}} \cdot \delta_a (\underline{V}_a \cdot \phi TV_L)^{\frac{2}{3}}$$

Substituting the function obtained for the correlation between body weight and TN_a, and also that obtained from the correlation between total alveolar volume ($\underline{V}_a = \phi \cdot TV_L$) and % TV_L, into Equ. 14, the total alveolar surface area (TS_a) is given by,

$$TS_a = \delta_a \cdot [10^6 \cdot [(0.0922 \text{ B.Wt.} + 10.47)]^{\frac{1}{3}} \cdot [0.1998 \% TV_L - 2.045]^{\frac{2}{3}}$$

The only unknown is the surface to volume ratio δ_a of the alveoli. This was found to have a mean value of 4.75 (see page 113)

Values for TS_a were computed using the above equation, for a range of values of % TV_L and for a range of values for body weight.

Fig. XXII shows the resulting curves as a series of body weight isopleths, with total alveolar surface area in cm² on the ordinate and percentage total volume of the lungs on the abscissa.

The values of TS_a measured by mean chord lengths, from all lungs examined, were then plotted on the B.Wt. / TS_a / %TV_L diagram. It was found that each value for TS_a in the control group lay very near the appropriate body weight isopleth. The values of TS_a and body weight given in Table XI are in order of increasing % TV_L and correspond to the control points on the B.Wt. / TS_a / %TV_L

TABLE XI - TOTAL ALVEOLAR SURFACE AREAS MEASURED
BY MEAN CHORD LENGTH

% TV _L	Body wt. g.	TS _a x 10 ⁻³ cm ²	Vent. time. min.
17.46	482.1	3.3104	0
28.45	442.5	3.7526	0
38.07	372.9	4.1352	0
45.13	358.2	4.3269	0
45.62	417.5	4.9073	0
56.66	430.5	5.7421	0
61.43	447.7	6.3391	0
71.56	737.8	11.2225	0
75.7	462.5	6.8708	1
14.21	581.5	2.2962	5
48.5	667.8	6.9267	5
62.5	486.2	5.4370	5
72.4	510.0	6.8121	5
15.1	640.2	2.8472	10
24.7	420.0	2.3525	10
46.2	360.0	3.4700	10
74.5	472.0	5.6513	10
20.4	545.0	2.6030	15
45.1	435.0	2.9528	15
69.7	470.0	4.6180	15
87.14	523.5	6.4279	15

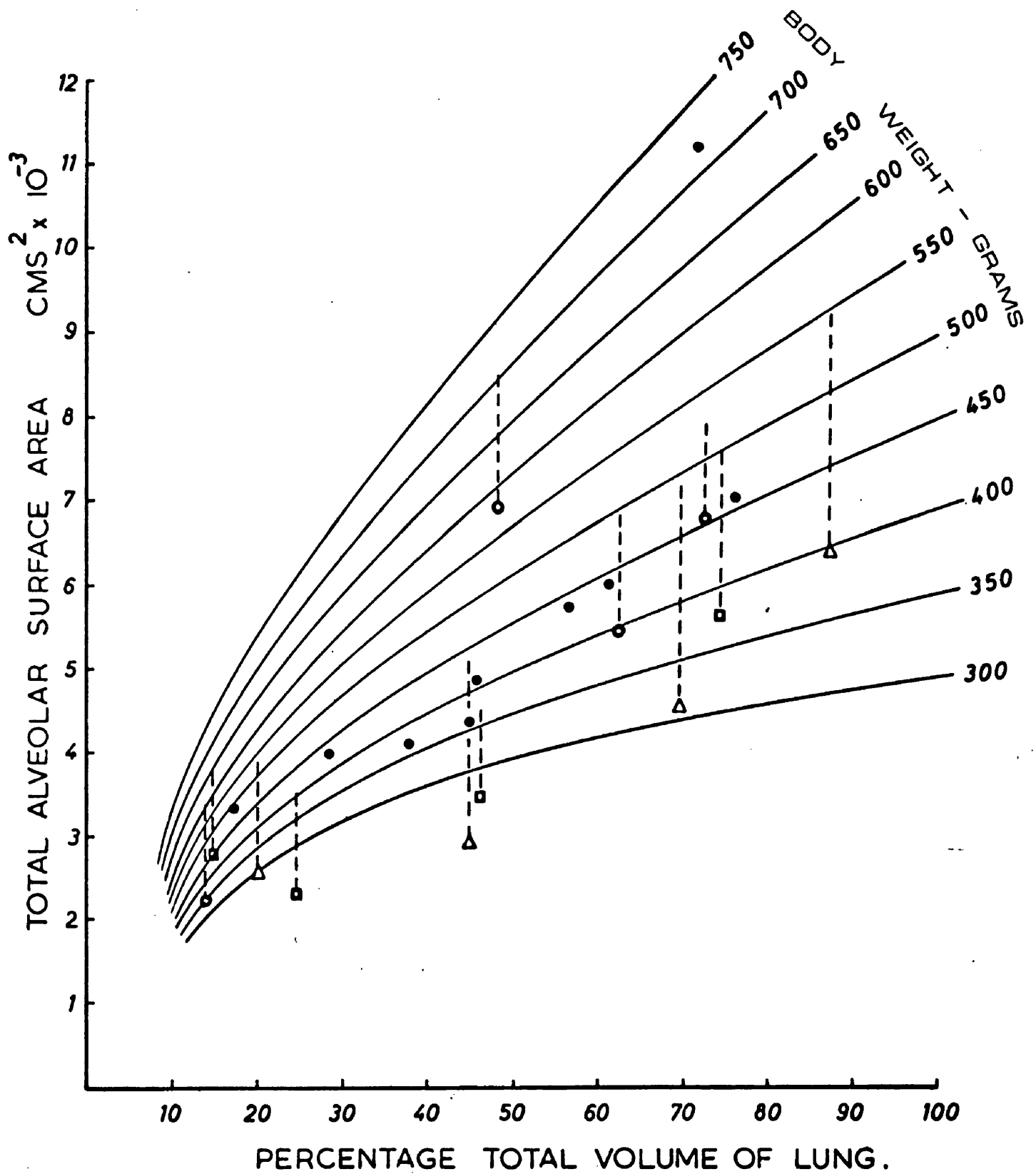


Fig. XXII - THE $TS_a / \%TV_L / B.wt.$ DIAGRAM

diagram reading from left to right. The actual weights of the control animals were not inserted in Fig. XXII for the sake of clarity.

The values of TS_a for lungs which had been hyperventilated were plotted on the $B.Wt. / TS_a / \%TV_L$ diagram. The vertical distance of each point below its appropriate isopleth is shown by the broken lines. This is a measure of the amount by which TS_a was reduced by hyperventilation. The absolute values by which TS_a was reduced are given below for each of the three hyperventilation groups, with the percentage reduction given in brackets.

HYPERVENTILATION PERIOD			
5 min	10 min	15 min	
1.15 (21%)	1.0 (25%)	1.35 (34%)	Reduction of TS_a in $cm^2 \times 10^3$ (% reduction)
1.6 (18%)	1.5 (28%)	2.2 (42%)	
1.4 (18.5%)	1.1 (24%)	2.66 (41%)	
1.15 (20%)	1.95 (25%)	2.85 (33%)	
1.4 (19.4%)	1.2 (25.5%)	2.53 (40%)	MEAN

It can be seen that the total surface area is reduced progressively with the duration of hyperventilation, so that after 15 minutes it is 40% less than the predicted control value.

SURFACE TO VOLUME RATIOS

The mean surface to volume ratios (= shape coefficients) were computed for each of the lungs studied using Equ. 13, and are given in Table XII. Fig. XXIII shows the mean shape coefficients plotted against $\%TV_L$. The values for shapes comparable to alveolar shapes are shown by the horizontal broken lines.

SHAPE COEFFICIENTS

Controls

Animal	% TV _L	Body wt. gms.	TS _a cm ²	TN _a x 10 ⁻⁶	TV _a cc	Shape Coeff. S _a
63/68:2	17.46	482.1	3780	56.2	2.6037	5.212
63/68:3	28.45	442.5	4466	50.45	3.9453	4.842
68/68:2	61.43	447.7	7735	57.1	8.5050	4.821
69/68:1	45.13	358.2	5280	42.3	6.0452	4.566
70/68:1	45.62	417.5	5939	47.2	7.0143	4.487
70/68:2	38.07	372.9	4920	38.3	4.9956	4.996
61/68:2	71.56	737.8	13686	77.2	16.3257	4.994
61/68:3	56.66	430.5	7014	61.1	8.0260	4.442

Hyperventilated for 5 mins.

63/68:1	62.5	486.2	6672	49.4	7.2257	4.864
11/69:1	72.4	510.0	8322	59.1	9.1904	4.870
11/69:2	48.28	667.8	8399	81.3	10.1591	4.134
11/69:4	48.5	428.0	4872	53.4	5.0984	4.368

Hyperventilated for 10 mins.

68/68:1	24.7	420.0	2690	42.3	2.1385	4.651
63/68:4	15.1	640.2	3119	69.6	1.8286	5.070
69/68:2	46.2	360.0	4206	48.7	3.8112	4.721
70/68:3	74.5	472.0	6904	55.8	8.4012	4.370

Hyperventilated for 15 mins.

59/68:2	20.4	545.0	3019	51.7	2.0808	4.972
77/68:1	45.1	435.0	3550	52.4	3.8399	3.869
61/68:4	69.7	470.0	5646	52.2	6.7884	4.215
11/69:3	87.14	523.5	7833	52.8	10.8678	4.257

TABLE XII

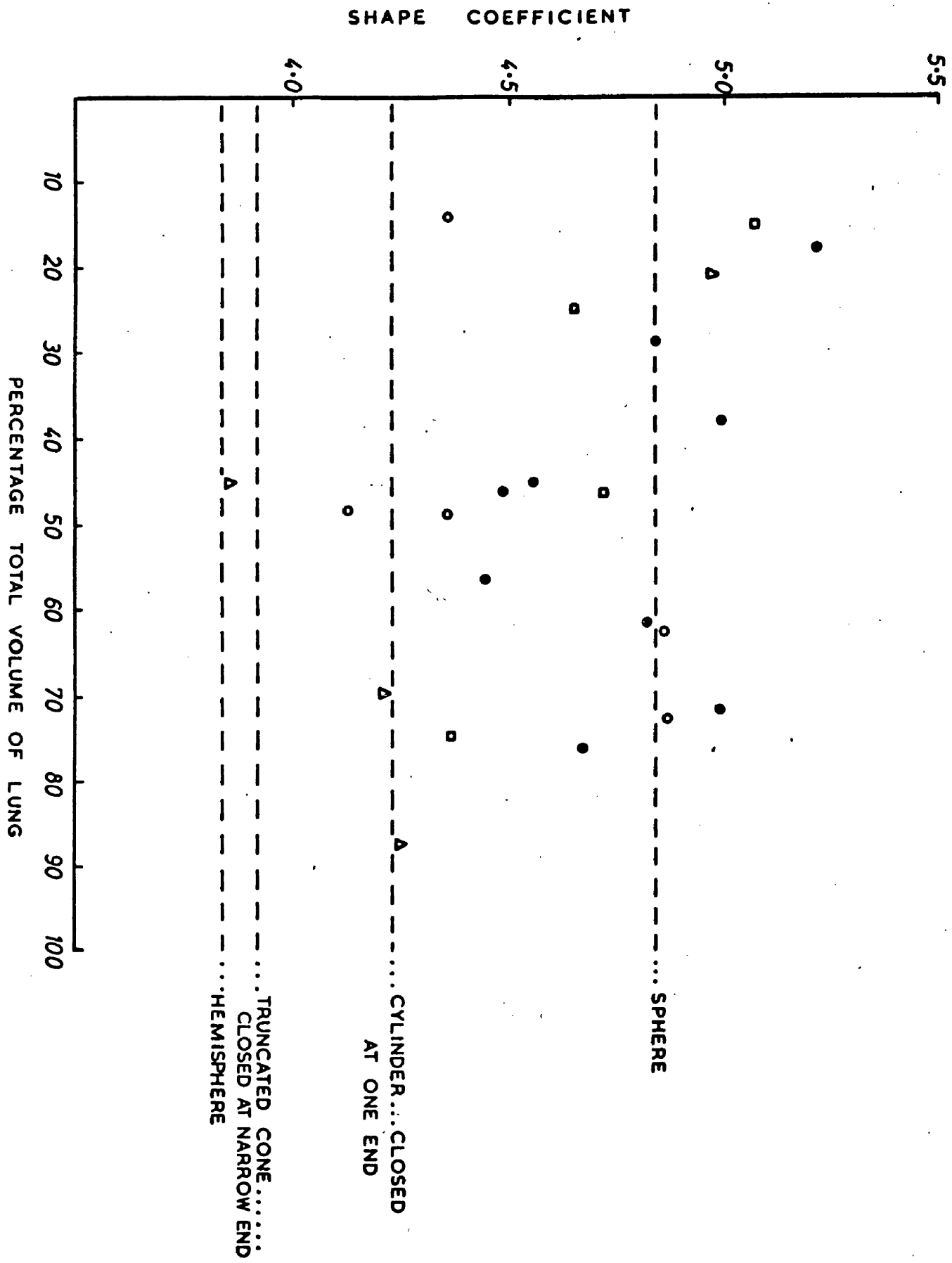


Fig. XXIII

No statistical correlation was found between the shape coefficient and $\%TV_L$; but there is a tendency for the shape coefficient to become less as the lung is inflated.

Hyperventilation tended to reduce the shape coefficient.

The overall mean value was found to be 4.75 (1 SD \pm 0.21). This corresponds to a shape approximately equal to 5/6 of a sphere.

LINEAR DIMENSIONS OF ALVEOLI AND ALVEOLAR DUCTS

The mean diameters of the alveolar mouths and the alveolar ducts are given in Table XIII. Fig. XXIV shows the mean alveolar mouth diameters \pm 1 S.D. and the mean alveolar duct diameters \pm 1 S.D., are plotted against $\%TV_L$, for each of the four ventilation groups.

It is clearly seen that the mean alveolar duct diameter increased with increase in lung inflation; from 700 μ at 20 $\%TV_L$ up to 1000 μ at 70 $\%TV_L$. No statistical difference was found between the four ventilation groups.

The mean alveolar mouth diameter increased with increase in lung inflation, but the exact relationship was not satisfactorily found because of the wide scatter of results.

Hyperventilation, caused the mean alveolar mouth diameter to be less than in the control lungs, but no difference was found between the means for the three hyperventilation groups.

TABLE XIII - MEAN ALVEOLAR MOUTH DIAMETERS AND MEAN ALVEOLAR DUCT DIAMETERS

% TV _L	Mean Alv. Mouth Diam. $\mu \pm 1 \text{ S.D.}$	Mean Alv. Duct Diam. $\mu \pm 1 \text{ S.D.}$	Vent. time min.
17.46	133 \pm 28	710 \pm 52	0
28.45	472 \pm 34	710 \pm 36	0
38.07	280 \pm 37	776 \pm 47	0
45.13	403 \pm 46	782 \pm 56	0
45.62	461 \pm 35	850 \pm 48	0
56.66	501 \pm 56	918 \pm 62	0
61.43	457 \pm 43	943 \pm 45	0
71.6	448 \pm 31	1017 \pm 51	0
75.7	554 \pm 49	1132 \pm 67	1
14.21	92 \pm 30	705 \pm 43	5
48.28	251 \pm 32	838 \pm 62	5
62.5	299 \pm 46	1004 \pm 78	5
72.4	348 \pm 38	1001 \pm 63	5
15.1	103 \pm 42	649 \pm 56	10
24.7	132 \pm 37	694 \pm 70	10
46.2	206 \pm 42	767 \pm 49	10
74.5	303 \pm 45	963 \pm 58	10
20.4	96 \pm 28	659 \pm 61	15
45.1	134 \pm 36	744 \pm 54	15
69.7	291 \pm 32	997 \pm 57	15
87.14	317 \pm 57	1242 \pm 76	15

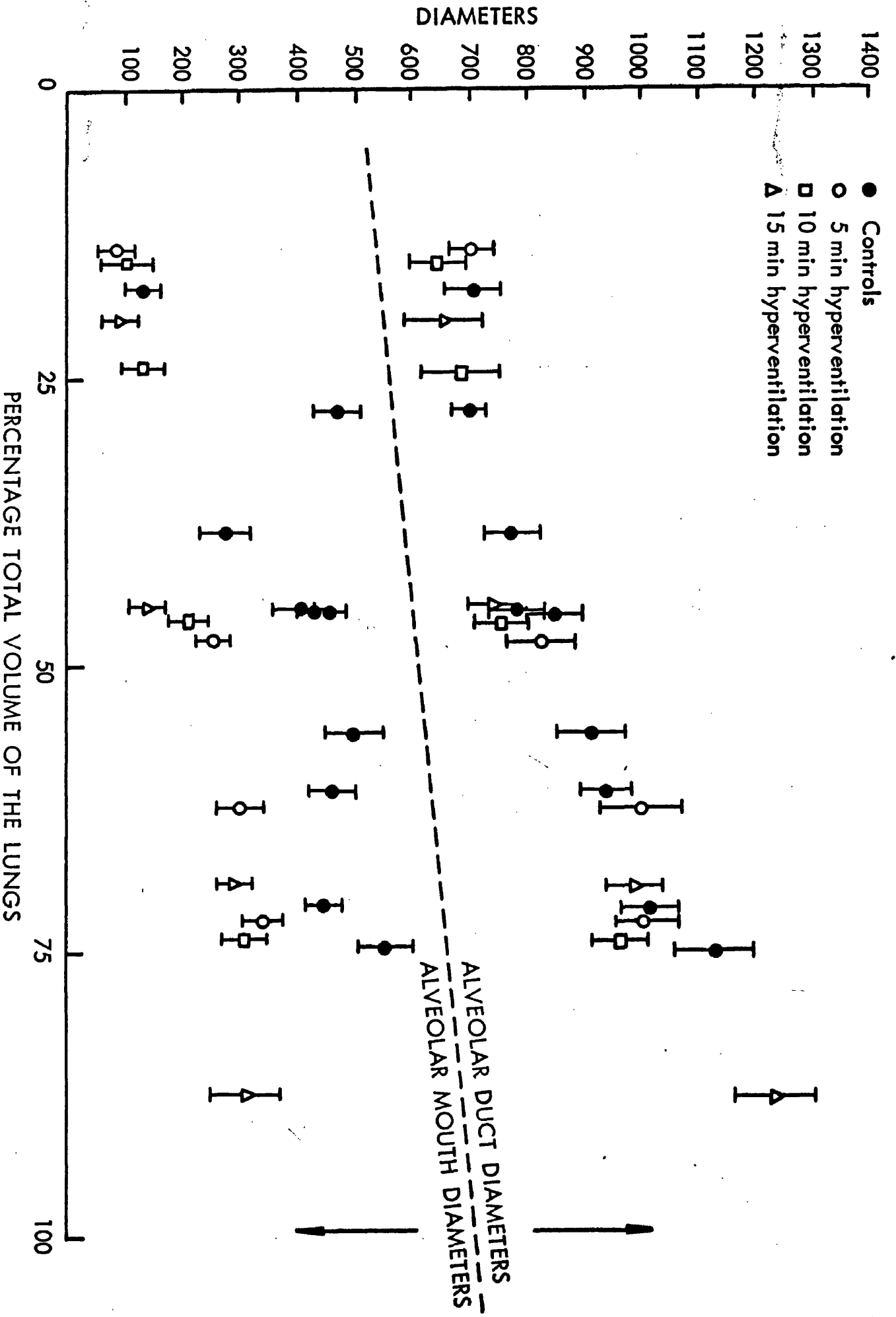


Fig. XXIV. Mean alveolar duct diameters \pm 1 S.D. above the broken line
 Mean alveolar mouth diameters \pm 1 S.D. below the broken line

ELECTRON MICROSCOPY OF INFLATED AND
COLLAPSED LUNGS

It was not possible to use for electron microscopy lungs which had been rapidly frozen and fixed by freeze substitution, since submicronic ice crystal formation caused loss of much of the normal electron microscopic appearance of intracellular constituents. Perfusion fixation with buffered gluteraldehyde gave excellent preservation of intracellular organelles, as well as the normal spatial cellular arrangement.

Observations:

The various cell types making up the alveolar wall were clearly defined (see Frontispiece and Plate XV). Abundant examples of the type II alveolar cell were seen at the corners of adjacent alveoli. Less frequently the type I alveolar cell nucleus was seen with its cytoplasm extending over the alveolar surface. Alveolar macrophages were occasionally seen floating free in the alveoli or lying on the alveolar surface, but separated from it by the cytoplasmic extensions of the type I cell.

The inclusion bodies (cytosomes) of the alveolar macrophage were similar to those within the cytoplasm of the type II cells (see later).

Large sheets of elastin were frequently seen in the alveolar wall. In most cases the elastin formed a cup around the type II cells, possibly serving as a protective shield preventing excessive deformation of the cell during lung inflation.

No difference in the intracellular appearance of the various alveolar cells was seen between the inflated and collapsed lungs.

Position of the type II cell in the alveolar wall:

The type II cells were mostly seen to be tucked deep into the wall of the alveolus. The cytoplasmic extensions of the type I cell abutted against them.

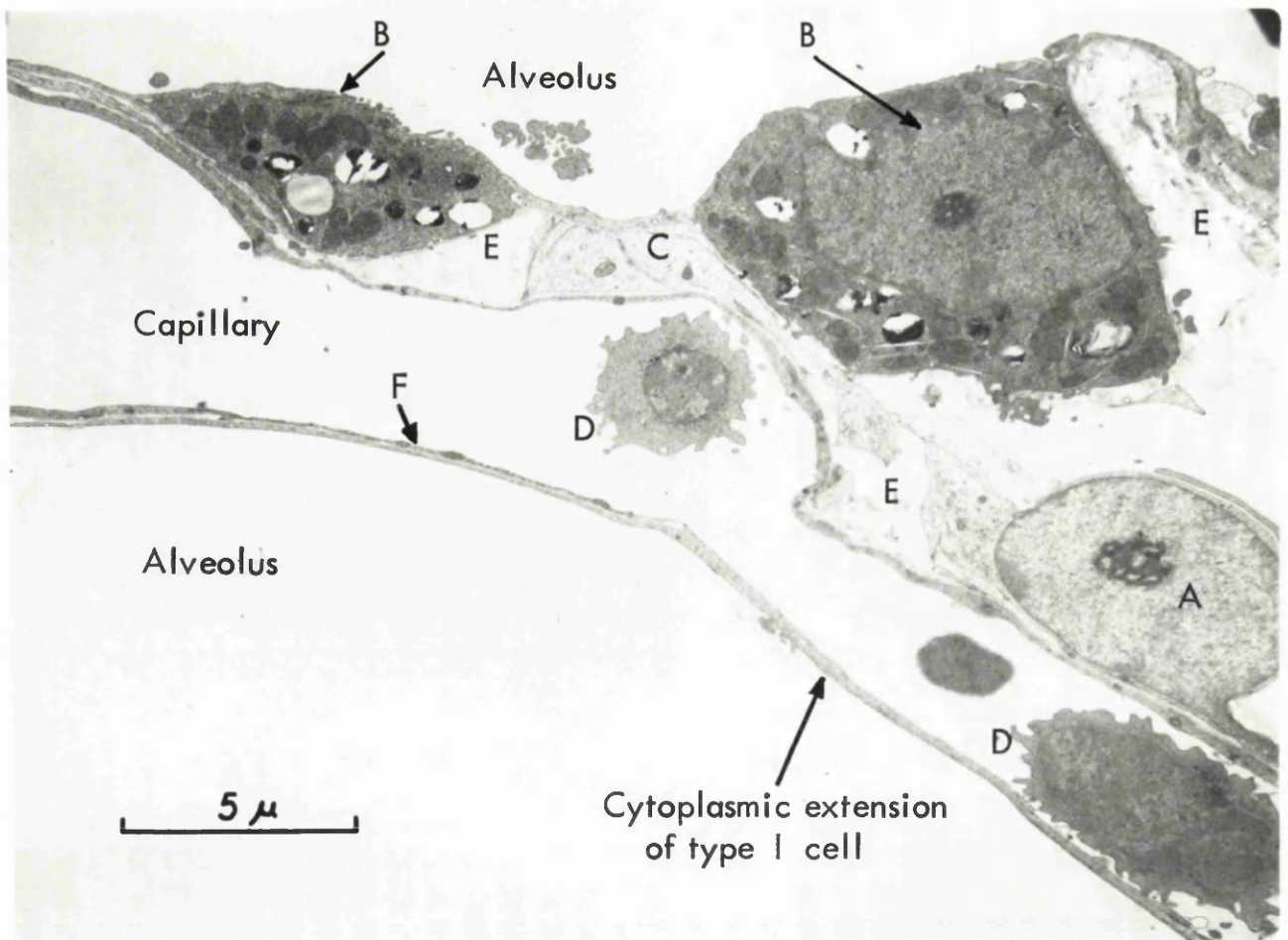


PLATE XV - The alveolar wall of expanded guinea pig lung ($\times 5,400$) showing the cell types making up the alveolar wall.

- A - Type I Alveolar pneumonocyte
- B - Type II Alveolar pneumonocyte
- C - Fibrocyte
- D - White blood cell
- E - Elastin fibres
- F - Endothelium

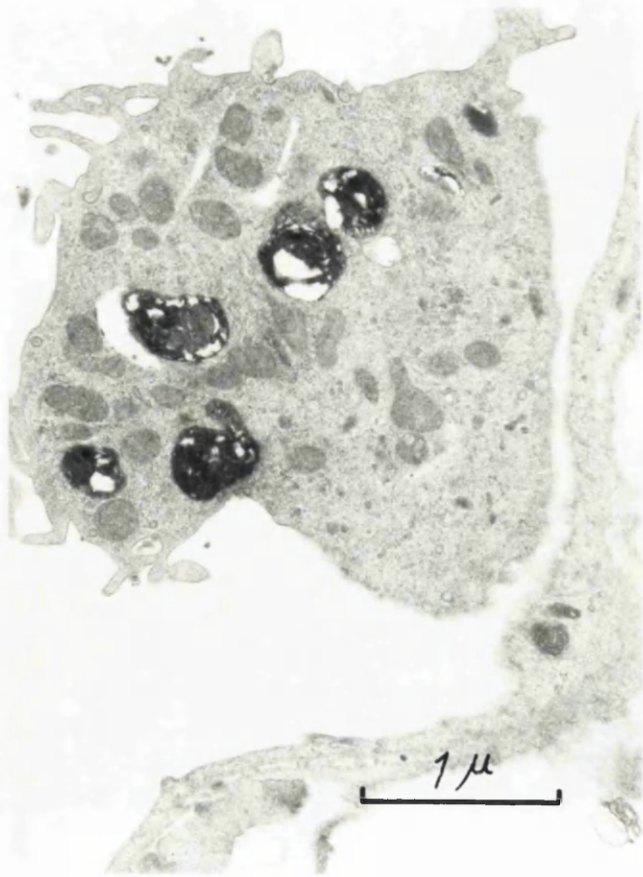
Plates XVIa - d show the four most common positions that the type II cells occupied. Plate XVIa shows the type II cell lying completely exposed on the alveolar surface. This cell is identified by the fact that there were no cytoplasmic extensions of the type I cell at the base of the type II cell. There appeared to be little adhesion between the type II cell and the underlying basement membrane. Plates XVI b and c show intermediate stages, where the type II cell is half buried in the alveolar wall. It can be seen that the microvilli extend over the whole exposed surface of these cells. Plate XVI d shows a type II cell almost completely buried in the alveolar wall, with only a small area of microvilli exposed on the surface. It is noteworthy that the number of inclusion bodies is greater the less exposed the cell.

It would seem to be possible that the series of plates XVI a - d not only represents the static spectrum of cell positions in the alveolar wall, but also might be an illustration of a possible turnover route for these cells, from d through c & b to a. This infers that as the cell ages the number of inclusion bodies diminishes.

Although many of the type II cells were examined carefully, no evidence was found to suggest that the osmiophilic inclusion bodies discharge their contents onto the cell surface. Abundant pinocytotic vesicles were seen on the surfaces of the type I, type II and capillary endothelial cells. There was a concentration of these vesicles at the microvilli of the type II cell suggesting that active transfer of 'substances' occurs here.

Osmiophilic inclusion bodies in the type II cell:

These bodies were typical of the type II cell. Plate XVII shows a high power electronmicrograph of two osmiophilic inclusions in a type II cell. It can be seen that a double layer plasma membrane surrounds each inclusion body. The substance within the inclusions was osmiophilic and is demonstrated to have a lamellar structure. The interval between lamella is about 50 - 100Å. This



a



c



b



d

PLATE XVI - Showing most common positions that the type II cells occupy

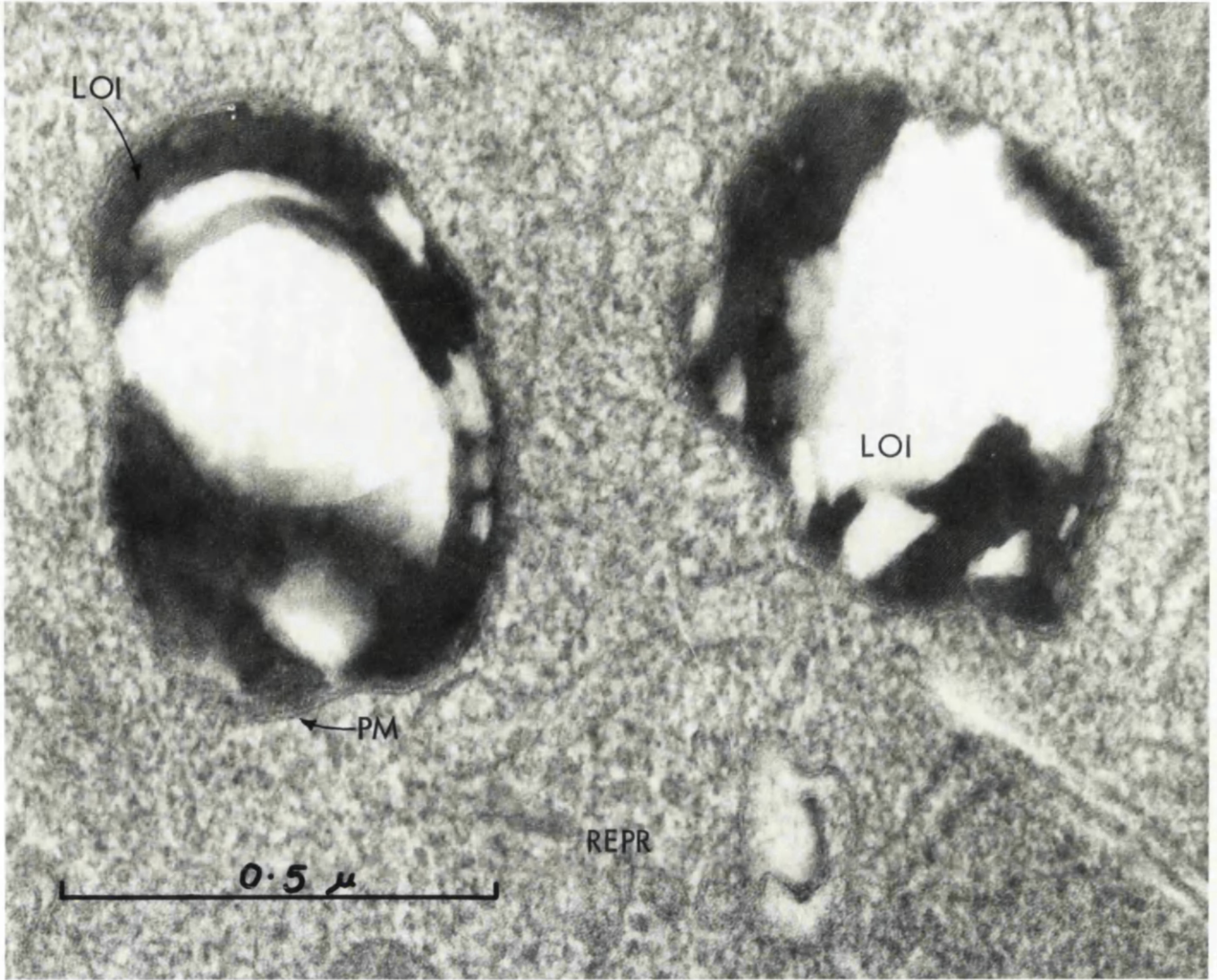


PLATE XVII - Lamellated osmiophilic inclusion bodies (LOI) in a type II alveolar pneumonocyte. Showing a double layer plasma membrane (PM) surrounding the inclusions and the abundant rough endoplasmic reticulum (REPR).

(magnification $\times 112,000$)

corresponds to the lamellar interval of lecithin (Bangham & Horne, 1964).

Abundant rough endoplasmic reticulum is also demonstrated.

Alveolar membrane:

Plates XVIII a & b show the alveolar membrane in the collapsed and inflated lungs respectively. In both cases the cytoplasmic extension of the type II cell is seen to be separated from the capillary endothelial cell by a thick band of intercellular substance. The junctional complexes between adjacent cells are clearly seen. It can be seen that these are tight bridges, It was observed in most of the sections examined from the inflated lungs that the junctional complex nearly always ran at right angles to the surface membrane (Plate XIII b). Whereas in the collapsed lungs the alveolar cell type I junctional complexes were most often seen lying at a tangent to the alveolar surface (Plate XVIII a).

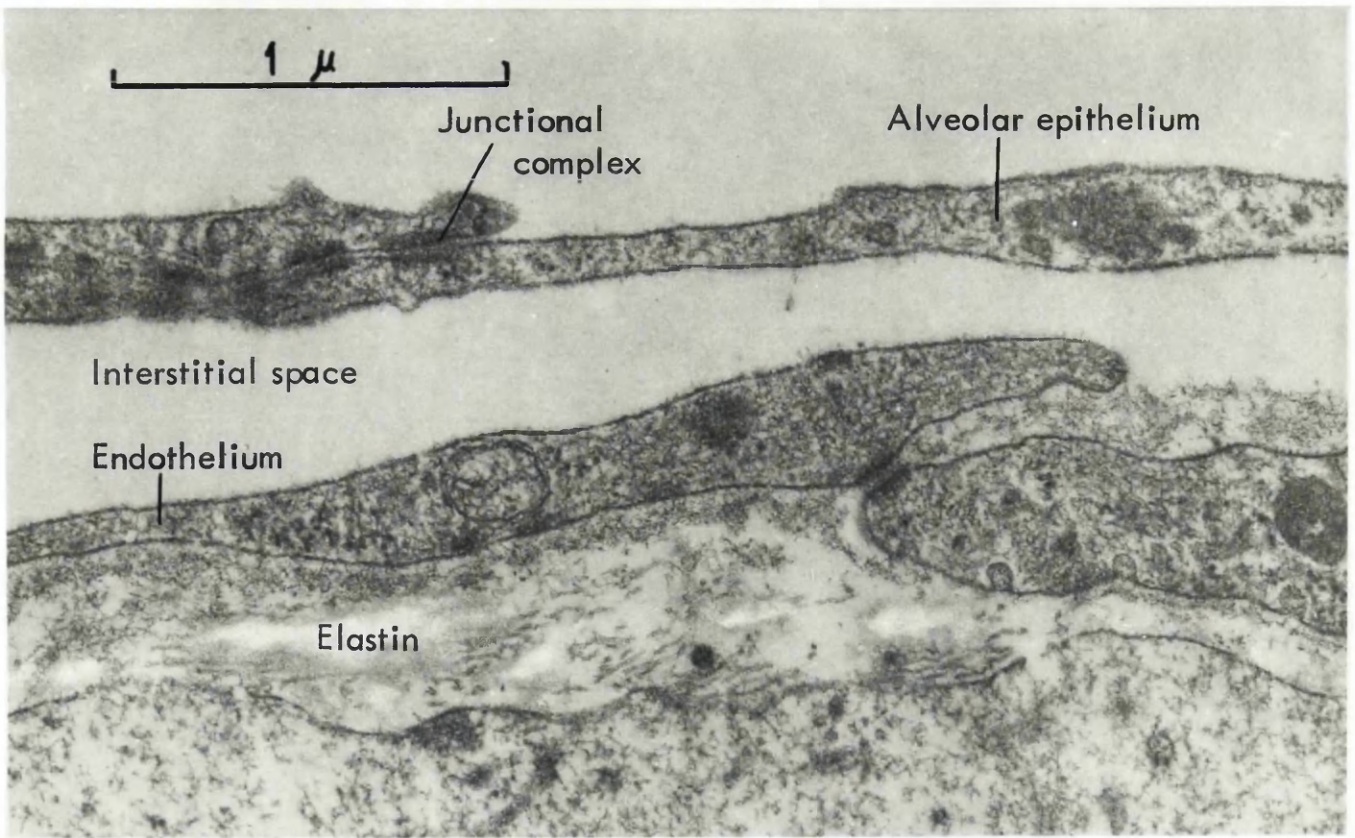
No evidence was found that the alveolar surface membrane folded when the lungs were collapsed. In both the inflated lungs and the collapsed lungs the alveolar membrane appeared as a smooth contour.

Osmiophilic electron dense material was occasionally seen lying on the alveolar surface (Plate XVII b). The possibility that this material was artifact cannot be ruled out; but it did not have the usual appearance of artifact. It may possibly have been small collections of the staining material lead acetate.

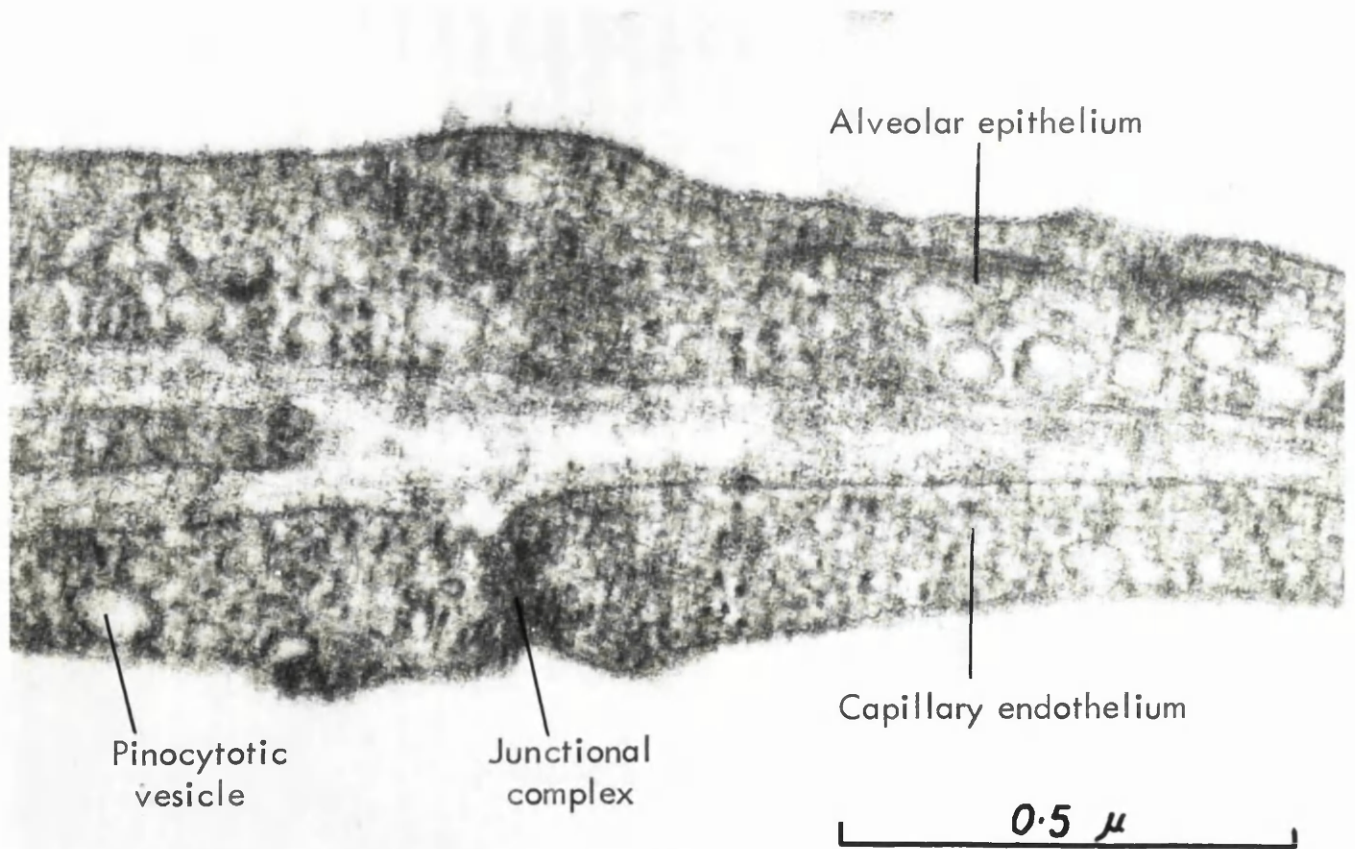
Air-blood barrier:

The harmonic mean thickness of the air-blood barrier was measured on 20 electronmicrographs of inflated lungs and on 20 of collapsed lungs. A total number of 460 estimations were made of the barrier thickness on the inflated lungs and 540 on the collapsed lungs. The harmonic mean thickness was given by,

$$\bar{D}_h = \frac{n}{\frac{1}{D} + \frac{1}{D} + \frac{1}{D} + \dots + \frac{1}{D} \text{ } n \text{ times}}$$



a - Alveolar membrane, collapsed lung (x 45,000)



b - Alveolar membrane, inflated lung (x 105,000)

where: n was the total number of estimations, and

D was the barrier thickness in \AA .

The mean harmonic thicknesses of the total air-blood barrier as well as the individual barrier components are given below in \AA .

	Total	Alv. Epith.	Intersitium	Endothelium
Inflated	4000	665	1335	2000
Collapsed	6000	2000	1500	2500

It can be seen that the harmonic mean barrier thickness increased from 4000 to 6000 \AA in the inflated and in the collapsed lungs respectively. It is also noted that the relative change was greatest in the alveolar cell thickness between the collapsed and the inflated states.

SUMMARY OF ELECTRON MICROSCOPIC OBSERVATIONS MADE IN THIS STUDY

1. No evidence was found to suggest that the type II cell discharges its cytosomal contents onto the cell surface.
2. The concentration of pinocytotic vesicles in the region of the microvilli of the type II cell, suggests very active transfer of unknown substances at this site.
3. The lamellar interval of the osmiophilic inclusion bodies in the type II cell confirms the suggestion that lecithin may be a constituent of the inclusions (page 16).
4. No surface corrugation of the alveolar membrane was seen.
5. The harmonic mean thickness of the air-blood barrier is decreased when the lungs are inflated.
6. The thickness of the alveolar cell (type I) was reduced to a greater extent in the inflated lungs than was the capillary endothelial cell.

SUMMARY OF THE EFFECTS OF LUNG INFLATION

1. The alveolar volume fraction (\underline{V}_a) increases in the mid-inflation range, then decreases back to the value for low lung volumes above 60% TV_L .
2. The alveolar duct volume fraction (\underline{V}_d) remains constant during lung inflation.
3. The ratio of TV_a / TV_d falls steadily during lung inflation up to 60% TV_L , thereafter it remains constant.
4. TV_a increases during lung inflation; but levels off above 80% TV_L .
5. TV_d changes little until the lungs are inflated to above 40% TV_L ; thereafter TV_d increases steeply.
6. During lung inflation, TV_a increases proportionately more than TV_d up to 40% TV_L above which TV_a and TV_d increase to the same extent.
7. Total alveolar surface area (TS_a) increases during lung inflation. The absolute values of TS_a depend on the body weight as well as the degree of lung inflation.
8. The surface to volume ratio of alveoli is independent of the degree of lung inflation.
9. The mean alveolar duct diameter increases by about 40% when the lung is fully inflated from a near collapsed state.
10. The mean alveolar mouth diameter increases during lung inflation.

SUMMARY OF THE EFFECTS OF HYPERVENTILATION

1. \underline{V}_a decreases progressively with duration of hyperventilation.
2. \underline{V}_d is not altered by hyperventilation.
3. TV_a is reduced by hyperventilation.
4. TV_d is not altered by hyperventilation.
5. The ratio of TV_a / TV_d decreases progressively with duration of hyperventilation. Hyperventilation caused the TV_a to increase linearly with TV_d during lung inflation.

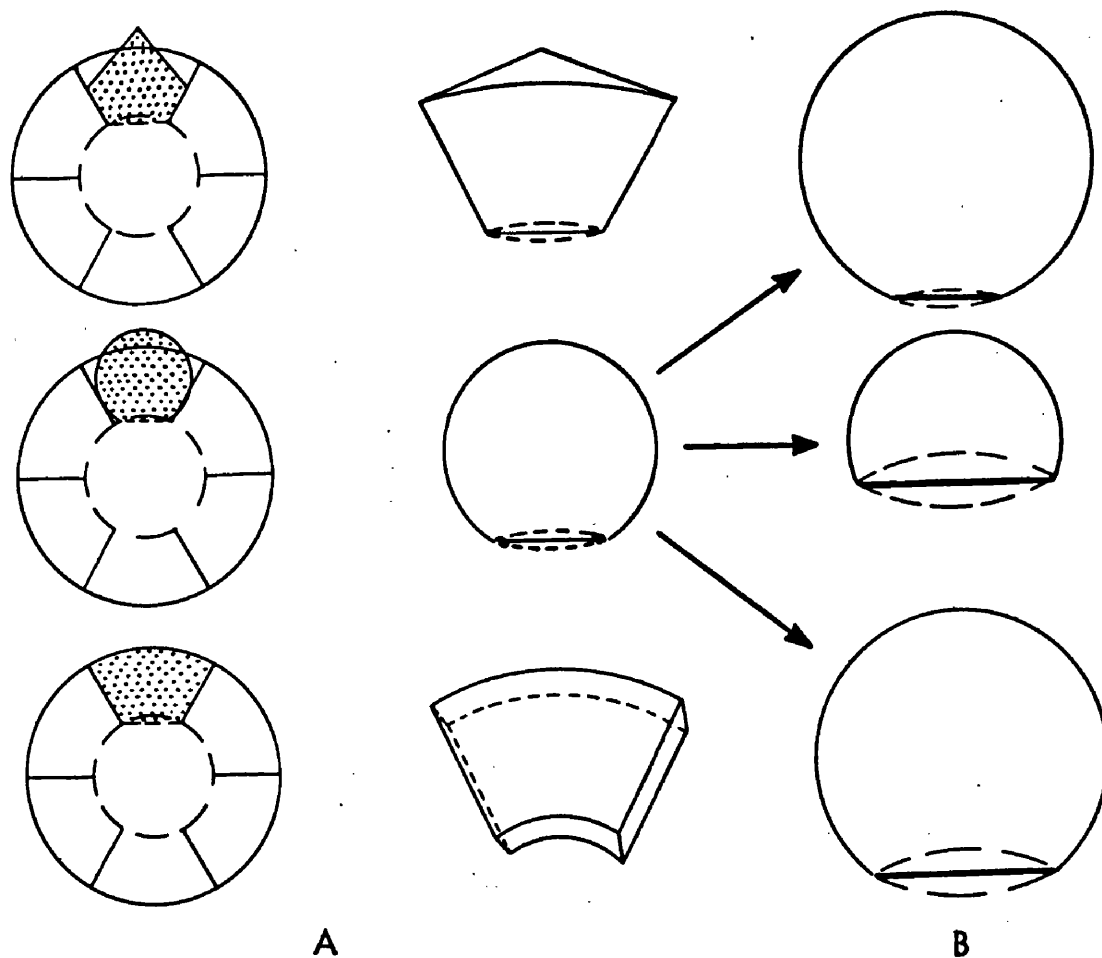


Fig. XXV. A. The more common alveolar shapes (after Weibel, 1963).
 B. The possible effects of lung inflation on a spherical alveolus.

6. TS_a was reduced after hyperventilation. The percentage reduction of TS_a increased with duration of hyperventilation and tended to be greatest at high degrees of lung inflation.
7. The alveolar shape coefficient tended to be reduced after hyperventilation.
8. The mean alveolar mouth diameter was reduced by hyperventilation.
9. The mean alveolar duct diameter was not altered by hyperventilation.

DISCUSSION OF MORPHOMETRIC ANALYSIS

It has been shown in the present study that during lung inflation the total alveolar volume and the total alveolar surface area increase. The increase was greatest from the low to the mid-inflation ranges. Above 80% TV_L there was little change in both TV_a and TS_a . The lack of a statistical correlation between the alveolar surface to volume ratios and degree of lung inflation indicates that during lung inflation there is little or no change in the basic shape of alveoli.

The increase in the mean alveolar mouth diameter during lung inflation need not alter the basic alveolar shape. Fig. XXV shows the more common alveolar shapes on the left (A), with the possible effects of lung inflation on the right (B), for a spherical alveolus. It is evident from the evidence produced in the present study that lower right (B), effect in Fig. XXV most nearly represents the effect of lung inflation on the alveolus; namely that alveolar volume, surface area and mouth diameter increase.

It has been suggested (Weibel, 1963a) that any change in alveolar surface area during lung inflation, might be due to unfolding of the alveolar surface membrane. Electronmicrographic evidence has been produced here indicating that the alveolar surface membrane is smooth at all degrees of lung inflation. This implies that it has inherent elasticity. Although the appearance of the junctional complexes (Plates XVIII a & b) suggests that there may be some degree of overlapping during collapse of the lung.

The increase in TV_d during lung inflation was greatest above 40% TV_L , in contrast to TV_a . This indicates that initially the forces which have to be overcome during expansion of the alveolus are less than those of the alveolar duct. Since the alveolar duct has a loose envelope of smooth muscle fibres, it is possible that these may hold the alveolar duct relatively stable initially. This suggestion may also account for the relatively small change in the mean alveolar mouth

diameter during lung inflation up to about 40% TV_L , since each alveolar mouth has a lip of smooth muscle fibres.

The initial proportionately greater increase in TS_a and TV_a during lung inflation could be accounted for by the presence of low surface forces in the alveoli at low lung volumes. This has been suggested by others from the surface tension properties of extracted lung material (see review of literature section on page 8 et seq.).

The relationship between TN_a and body weight contrasts with the findings of others (Weibel, 1963a); but the highly significant statistical correlation found in this study suggests that TN_a is directly related to the body weight. This suggests that TN_a may be determined by the metabolic needs of the individual. Functionally this would be more efficient than merely increasing the mean size of the alveoli. The work done in overcoming the surface forces of a large number of small alveoli would be less than that of a slightly smaller number of large alveoli provided that the surface tension is lower in small alveoli than in large ones.

The increase in the harmonic mean thickness of the air-blood barrier as well as the increase in the alveolar surface area during lung inflation, would facilitate the transfer of respiratory gases in the lung. Since oxygen consumption is related to body weight, it is perhaps not surprising that TS_a was found to be related to body weight.

Only one other study has been made on the effects of lung inflation. Dunnill (1967) found that TS_a was related linearly to the ratio of lung volume to lung weight. He also showed that \underline{V}_a increases, and \underline{V}_d decreases linearly with increase in the lung volume to weight ratio. Further useful comparison with this study would be very limited for the following reasons: the actual degree of inflation was not measured, the methods of fixation were entirely different from that of the present study, no correction was made for shrinkage due to processing

and finally the relationship between TS_a and body weight was not explored.

One would perhaps want to correlate the findings presented in this study in guinea pig lungs to similar changes in human lungs. The anatomical differences between the two were studied. Fig. XXVI shows the terminal unit of the guinea pig lung, drawn from 100μ thick serial lung sections, compared with that of the human lung. The terminal unit of the guinea pig lung differs from that of the human lung in several respects.

1. The respiratory bronchioles are shorter relative to the alveolar ducts.
2. The alveolar ducts are relatively longer and only infrequently branch beyond the first division at the respiratory bronchiole.
3. There are fewer alveolar sacs per alveolar duct.
4. The alveolar ducts frequently extend right up to the pleural surface.

Hyperventilation decreased \underline{V}_a but not \underline{V}_d , and TV_a but not TV_d , suggesting that the alveolar forces which have to be overcome during lung inflation were increased. The lack of effect on TV_d implies that tissue forces were largely unaltered following hyperventilation. This suggests that there was a change in alveolar surface properties such that surface tension forces were increased. This is also suggested by the finding that TS_a was reduced after hyperventilation.

In contrast to this however is the finding that the mean alveolar mouth diameter was decreased following hyperventilation. This suggests that the smooth muscle ring around the alveolar opening may have increased its tone. If surface tension forces were increased in the alveoli after hyperventilation, then this would tend to extend any opening in the alveolar wall. However it is possible that the decreased alveolar size after hyperventilation could by itself have caused the reduction in the alveolar mouth diameter. This implies that the forces determining the size of the alveolar mouth, act from within the alveolus and not from the alveolar duct.

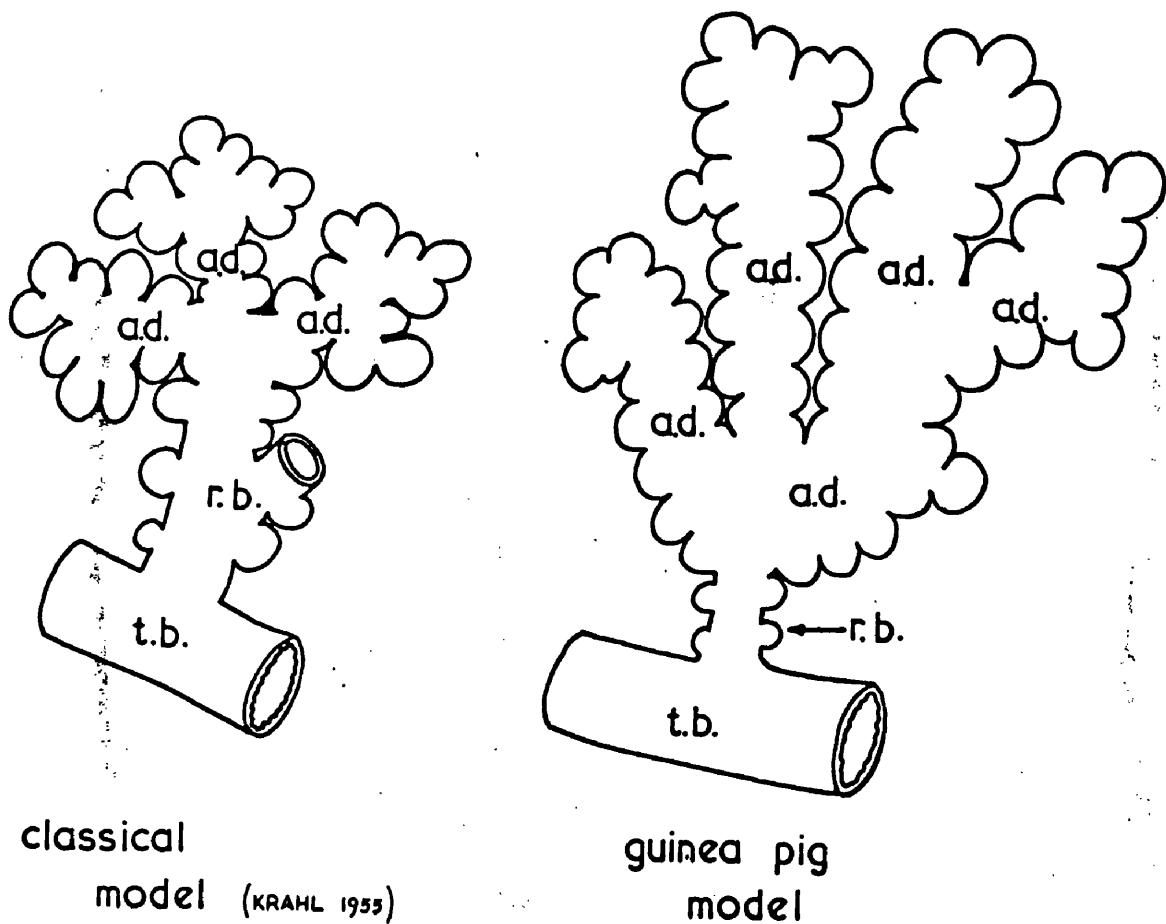


Fig. XXVI. Terminal lung units of human and guinea pig lungs.

GENERAL DISCUSSION

Evidence was presented in Part I of this study indicating that hyperventilation caused a significant change in the surface tension properties of the guinea pig lung. These changes were shown to be related to the duration of hyperventilation.

The pressure-volume relationship was altered after hyperventilation such that the lung tended to empty prematurely. The stability ratio of lung bubbles was decreased following hyperventilation, causing them to reduce in size more rapidly than the controls. Both findings suggest that the surface forces in the lung were increased.

The morphometric evidence presented in Part II, indicates that the main site of the changes following hyperventilation is in the alveolus. The increase

in TS_a during lung inflation meets the basic requirement of the theory of action of pulmonary surfactant: namely that surface tension in the lungs is dependent on surface area. At low lung volumes with reduced surface area, the surface tension would be low, thereby tending to maintain the stability of the alveolus.

After hyperventilation the alveoli were less expanded, and the surface area less than in the controls, indicating that there were increased surface forces, since the pressure used to inflate the lungs was equivalent in all animals.

The physical effect of hyperventilation on pulmonary surfactant and the way by which it causes an alteration to the surface tension properties, is not known. It is apparent that hyperventilation in some way degrades pulmonary surfactant and that this reduction in surface activity produces instability of the alveoli with a tendency to atelectasis.

Future Work:

More work is needed before the origins of pulmonary surfactant can be defined, and before its true functional significance can be identified. One would like to explore further the relationship between the total number and surface area of alveoli, and body weight. Little mention has been made of the other side of the coin, - the capillary system in the lungs. The change in capillary blood volume during lung inflation, the factors controlling the distribution of blood flow, and even more basically, the direction of flow at the alveolar level, all need careful investigation.

Lastly, one hopes that by defining the factors which affect pulmonary surfactant, the time may be brought nearer when the many pathological conditions associated with its depletion can be successfully treated.

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APPENDIX 1

STAINING METHODS

HAEMALUM & EOSIN

1. Bring section to xylol 5 min
2. Transfer to meth. spirit 3 min
3. 50% alcohol 3 min
4. water 5 min
5. haemalum..... 3 min
6. water..... 5 min
7. eosin 1 min
8. Rinse in water 1 min
9. Clear in meth. spirits..... 1 min
10. Transfer to abs. alcohol 1 min
11. carbol.Xylol..... 1 min
12. Xylol 2 min
13. Mount in Canada Balsum

PERIODIC ACID-SCHIFF (PAS)

1. Bring sections to water.
2. Rinse in 70% ethanol.
3. Immerse in periodic acid solution 5 min
4. Rinse in 70% ethanol.
5. Immerse in reducing bath 1 min
6. Rinse in 70% ethanol.
7. Immerse in Schiff solution 20 min
8. Wash in running water ... 10 min
9. Stain lightly with celestian blue..... 2 min
followed by Mayer's haemalum 2-3 min
10. Differentiate in 1% acid ethanol.
11. Wash in running water 30 min
12. Counter stain with Orange G 10 sec
13. Wash in water until the sections are pale
yellow about 30 sec
14. Dehydrate through the alcohols, clear in xylol
and mount in DPX.

GORDON & SWEET'S METHOD FOR RETICULIN

1. Bring sections to water.
2. Oxidise in acidified permanganate 5 min
3. Wash well in water.
4. Bleach until white in 1% oxalic acid.
5. Wash in running water.
6. Mordant in 2.5% aqueous iron alum 20 min
7. Wash in distilled water.
8. Impregnate in diamine silver hydroxide 1 min
9. Wash in distilled water.
10. Reduce in 10% formalin.
11. Wash in distilled water.
12. Tone in 0.2% gold chloride 3 min
13. Wash in distilled water.
14. Fix in 5% sodium thiosulphate 5 min
15. Wash in water, dehydrate, clear and mount in Canada Balsum.

BAKER'S ACID HAEMATIN METHOD

1. Cut frozen sections and mordant in dichromate-calcium for one hour at 60°C.
2. Wash in distilled water.
3. Transfer to acid haematin for five hours at 37°C.
4. Rinse in distilled water.
5. Transfer to borax-ferricyanide solution for eighteen hours at 37°C.
6. Wash in water.
7. Mount in glycerine jelly.

PHOSPHOMOLYBDIC ACID METHOD (LANDING)

1. Dry section thoroughly in air.
2. Dip into 50/50 acetone-ether.
3. Transfer to 1% phosphomolibdic acid in 50/50 ethanol-chloroform for 15 min.
4. Rinse in ethanol-chloroform, then in chloroform and dry.
5. Dip into 1% aqueous stannous chloride in 3-N HCl.

6. Wash in water.
7. Counter stain with 1% aqueous eosin.
8. Dehydrate, clear and mount in Canada Balsum.

FLUORESCENCE METHODS

1. Cut frozen sections.
2. Stain sections for 3 min. in 0.1% aqueous Phosphine 3R (or aqueous Rhodamine B).
3. Rinse in water 10 sec.
4. Mount in 90% glycerine.
5. Examine immediately with an ultra-violet microscope.