ABSTRACT

THE EFFECTS OF VARYING PHYSICAL ACTIVITY LEVELS ON: PULMONARY SURFACE ACTIVITY, GREAT ALVEOLAR CELL TO ALVEOLI RATIOS, AND GREAT ALVEOLAR CELL OXIDATIVE ENZYME HISTOCHEMISTRY

by Robert Echt

There is now abundant evidence demonstrating that pulmonary alveoli are lined with an extracellular layer (surfactant) which lowers surface tension and prevents collapse of the lungs and transudation of fluid from the capillaries.

However, a full understanding of the processes involved in surfactant synthesis and turnover is still lacking.

Experiments by Faridy et al. (1966) demonstrated that acute, rapid inflation and deflation of isolated adult rat and human lungs resulted in a brief loss of alveolar stability. With optimum oxygen tensions, temperature and pH, recovery was rapid. These events suggested an active process of surfactant production.

In vitro studies by Heinemann (1967) indicated that pulmonary surfactant production in vivo may be sensitive to sympathetic and parasympathetic stimuli, and to narrow ranges of oxygen tension.

Few investigators have observed the effects of prolonged physical exercise on pulmonary surface phenomena, proposed cellular sites of surfactant synthesis, and possible mechanisms for surfactant turnover.

The purpose of this study was to provide additional information for the following questions:

1. Are active surfactant depletion-regeneration phenomena effective in maintaining similar degrees of alveolar stability in animals exposed to increasing levels of physical exercise?

2. Does the *in vivo* maintenance of adequate surfactant concentration in chronically exercised animals produce significant increases in numbers of great alveolar cells per unit of lung space?

3. Do varying levels of physical activity alter histochemical reactivity of some great alveolar cell enzymes believed to be important in surfactant production?

To investigate possible relationships existing between varying physical activity levels and pulmonary surface activity, 60 adult, male rats were randomly placed into three groups. Control animals were housed in sedentary cages. A second group was placed in sedentary cages and subjected to a 30 minute swimming period daily. A third group had two 30 minute swimming periods daily and free access to voluntary activity wheels. Each animal in the latter two

groups had lead weights equal to three per cent of the body weight attached to its tail during the swimming periods. After 52 days, the animals were sacrificed and their lungs prepared for bubble stability measurements; determinations of great alveolar cells to alveoli ratios; and analyses of great alveolar cell oxidative enzyme histochemistry.

No significant differences were obtained for bubble stability ratios, and great alveolar cell to alveolar ratios in the three treatment groups.

Subjective evaulations of histochemical activity indicated increased intensities with increased physical activity for a few enzymes associated with surfactant synthesis.

The data received from all methods used in this study reflect the ability of healthy lung to maintain gradation of normal surfactant and alveolar stability despite stressful exercise regimens.

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Anatomy

1968

DEDICATED TO:

MY WIFE AND CHILDREN:

For their love and understanding

ACKNOWLEDGMENTS

The author wishes to express his deep and sincere appreciation to Dr. Rexford E. Carrow for his guidance, consideration, and friendship throughout this study, and for the use of laboratory equipment and supplies.

Words can only be symbolic of the gratitude expressed to Dr. William H. Knisely for his continuous encouragement, inspiration, and help.

Appreciation is also extended to Drs. M. Lois Calhoun, R. A. Fennell, and W. VanHuss for serving on the guidance committee and taking the time to read this manuscript.

Special thanks are due to my colleagues LeRoy Gerchman and Reggie Edgerton for their valuable advice and friendship.

The author is deeply indebted to Mr. J. Irvin Nichols, Executive Director, and to the Board of Trustees of the Michigan Tuberculosis and Respiratory Disease Association for their kind consideration and fellowship support.

A note of appreciation to Drs. M. Lois Calhoun and Bruce Walker for use of departmental space and equipment.

Thanks are due to Drs. W. VanHuss and W. W. Heusner for use of their laboratory facilities.

Gratitude is also expressed to Mrs. Barbara Wheaton and Miss Trudy VanHuss for their technical assistance.

Special thanks to Mrs. Elizabeth Mautz for all her help.

Deepest appreciation is extended to Mrs. Vinny Gerchman, and Mrs. Myra Wendell for the long hours spent typing this manuscript.

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INTRODUCTION

During the past few years, a number of experiments have provided greater insight into some of the processes involved in pulmonary surfactant formation and turnover. Many of these studies have measured the effects of acute physiological stress on excised mammalian lung tissue.

Although much important information regarding alveolar surface-active material has recently been advanced, a full understanding is still lacking concerning the sites for synthesis and the metabolic processes associated with the elaboration of this lining.

In separate studies, Faridy *et al*. (1966) and Gruenwald (1966) reported that acute, rapid inflation and deflation of isolated adult rat and human lungs resulted in a brief loss of normal stability of expansion. Recovery became more rapid as physiological ranges of both temperature and oxygen tension were reached. These events suggested an active process of surfactant production.

In vitro studies by Heinemann (1967) on lung tissue indicated that insufficient or excessive amounts of: oxygen, epinephrine, norepinephrine, acetylcholine, and pre-treatment with resperine limited acetate incorporation into lipids. He concluded that synthesis of specific lipids of the

alveolar lining layer might therefore be sensitive to a narrow range of oxygen tensions and to sympathetic as well as parasympathetic stimuli.

With these studies in mind, an attempt was made to estimate pulmonary surface activity in three groups of adult male rats subjected to varying levels of prolonged physical activity.

In conjunction with estimations of surface activity, the following questions were also asked:

1. Are active alveolar lining depletion-regeneration phenomena effective in maintaining a degree of alveolar stability in severely exercised animals comparable to the stability in animals exercised less strenuously?

2. Does the maintenance of adequate surfactant concentrations in chronically exercised animals influence significant changes in numbers of great alveolar cells per unit of lung space?

3. Are these indications of an increase or decrease in the histochemical reactivity of some alveolar cell enzymes believed to be important in surfactant production?

REVIEW OF LITERATURE

PULMONARY SURFACE PHENOMENA AND LUNG MECHANICS

According to Mead (1961), Donders in 1849 was the first to note that lungs collapsed when the thorax was opened. Inherent pulmonary elastic retraction was responsible for the phenomenon. This retractive force, measured in terms of airway pressures, increased as the lungs were inflated (Mead, 1961).

Heynsius in 1882 found variable results when he measured retractive pressures at various volumes (Mead, 1961). He reported that if the lungs were allowed to collapse, re-expansion was not even and the number of alveoli open at the same air volume determined the magnitude of tracheal pressure. The volume-pressure curves obtained by Leibermeister in 1907 for excised cat and human lungs inflated from the collapsed state indicated that changes in volume were small until pressures of 8 to 10 cm H_2O were reached. After these levels, lung filling was large with subsequent small increases in pressure. Leibermeister related this to air passage closure in collapsed lungs being reversed as pressure increased. The increased pressure

re-established continuity between the trachea and the air spaces (Mead, 1961).

More recent investigators using inflation and deflation measurements in various mammals (McIlroy, 1952, Radford, 1957) found smaller pressures during deflation. They demonstrated that the complete volume-pressure cycle, which included inflation and deflation measurements, formed a hysteresis loop. Hysteresis and hysteresis loop were defined by Landowne and Stacy (1957) as follows: "Hysteresis is the failure of a system to follow identical paths of response upon application and of withdrawal of a forcing agent. The result of this failure to retrace the same path on withdrawal as on application is the formation of a hysteresis loop."

The contribution of pulmonary alveolar surfaces to lung function was first described by von Neergard (1929). He considered the surface geometry within the lungs to be analogous to the formation of a bubble on the end of a capillary tube. Using this model, he suggested that alveolar surface tension was an important component of the retraction pressure of the lungs. Since his findings, it has been shown (Clements, 1956, Brown, 1957, Brown et al. 1959) that surface phenomena exert major influences on lung hysteresis.

A bubble on the end of a capillary tube was regarded by von Neergard (1929) as a segment of a sphere which exerted a pressure as given in the Laplace relationship $P=2\gamma/r$. "If

r_1 and r_2 are the principal radii of curvature at a point on the surface, the pressure difference across the surface (P), is related to these radii and the tension in the wall (T), by Laplace's expression: $P=T(\frac{1}{r_1}+\frac{1}{r_2})$. For a spherical segment, $r_1=r_2=r$, and $P=\frac{2T}{r}$; for a cylindrical section where $r_1=\infty$, $P=\frac{T}{r}$. For a bubble with a single surface $T=\gamma$, where $\gamma=$ surface tension." (Mead, 1961).

Von Neergard's work precedes the discovery of the substance responsible for lowering surface forces in the lung. He compared volume-pressure curves after air filling with curves obtained after liquid filling in order to measure surface tension effects on pulmonary retraction pressure. Fresh, excised lungs, were initially inflated to nearly their maximum volumes. Static deflation curves were obtained. The gas was then removed from the lungs by evacuation in a vacuum jar. Next, the lungs and the chamber containing them were filled with a 7% gum arabic solution and then emptied in order to obtain a stepwise volume-pressure curve. Von Neergard found the transpulmonary pressures lower for a given volume of fluid than for air. Essentially the same relationships were found for curves obtained on sheep, pig, and human lungs. He concluded that:

1) Two-thirds to three-fourths of the total pulmonary retractive pressure was due to interfacial forces;
2) at normal end-expiratory levels, tissue retraction approached zero;

3. surface retractive pressures increased with increasing lung volumes (He suggested that the degree of internal curvature of the lung increased with lung volume and this resulted in increased surface retractive pressures.);
4) the surface tension at the alveolar surface might be lower than that of other physiological fluids as a result of higher concentrations of surface-active materials accumulating at the surface.

To support his assumption, he made surface tension measurements on lung extracts, reasoning that any surface-active material would move to the surface of these extracts. He found surface tension values of 35 to 51 dynes/cm [surface tension which is a force/distance is measured in units of dynes/cm (Comroe, 1965a)].

Von Neergard's proposals received little attention until Mead, Whittenberger, and Radford (1957) obtained a complete range of pressure-volume measurements from saline and air-filled dog lungs. Their emphasis on the problem differed from von Neergard's approach. They designed experiments to provide information about the marked hysteresis in air-filled lungs when compared to saline-filled lungs. Saline filling minimized surface activity and hysteresis loops were small, thus, the relationship between pressure and volume on inflation was approximately the same as that

obtained on deflation. Expansion in air-filled lungs was not uniform, and hysteresis was marked.

They also found that the transpulmonary pressure measured under static conditions was greater at a given volume when it was reached from a less inflated state than the pressure at the same volume after full inflation.

These workers concluded that resistance to flow was not responsible for differences in the hysteresis loops obtained, but due, rather, to surface tension exerting its influence on the distribution of air into a previously airless lung.

Gruenwald's (1947) earlier work suggested the importance of pulmonary surface forces on human fetal lung expansion uniformity at the time of birth. He found excised, air-inflated, neonatal human lung was under greater pressure than liquid inflated lungs. He believed that surface tension accounted for the higher inflation pressures required in air-filled lungs.

Radford (1954) postulated that evaluation of the differences in the air and saline pressure-volume curves allowed for measurement of surface area of the air spaces if a value for surface tension were assumed. (He used the surface tension of serum which is about 50 dynes/cm.) Surface energy considerations were employed in his approach to the problem. The free energy in a surface under constant tension is equal

to the product of surface area and surface tension. He assumed that:

1) The area between fluid and gas volume-pressure curves represents dissipation of free energy from the surface;
2) the tissue retractive pressures are the same function of volume in both instances;
3) none of the air spaces closes off until total deflation occurs.

Using this method, Radford obtained values for rat, cat, dog and human lungs. He calculated that the surface area at functional residual capacity was somewhere between 5 and 10 m^2 for a 70 kg adult human. This derived surface area was about one-tenth the value estimated from anatomic measurements. In discussing this difference, he suggested the discrepancy was probably due to errors in anatomic measurements and not due to his assumed surface tension value.

A few years after Radford's experiments, Brown (1957) and Brown *et al*. (1959) reported that the volume-pressure relationships of bubbles formed by blowing nasal mucus and pulmonary edema fluid on a tube showed marked surface tension decreases during deflation. He measured changes of surface tension from a high of 40-50 dynes/cm in a large bubble to a low of 5-10 dynes/cm when the bubble retracted.

He later used Radford's (1954) methods for measurements of fluid and gas-filled lungs to calculate surface tensions. At full lung inflation, he assumed surface tension to be 50 dynes/cm. From the total area calculated for these lung levels and the assumed area-volume relationship, he estimated area change at lower volumes. With the related surface energy changes, he then could estimate the surface tension at any volume. The values obtained for surface and surface tension relationships were the same for the pulmonary edema and nasal mucus bubbles. The surface tension at low lung volumes ranged between 5 and 10 dynes/cm.

Pattle (1955, 1956, 1958) in studies of antifoaming agents on tracheal and bronchial foam associated with acute lung edema presented further evidence of low surface tensions in the lung. His observations have provided valuable information regarding the nature of the alveolar lining.

Nickerson and Curry (1955) reported that use of an aerosol of silicone anti-foaming agent had a therapeutic effect on tracheal and bronchial foam found in acute lung edema. Pattle (1958), however, found that such foam was unaffected by these silicone anti-foaming agents or by octyl alcohol. The antifoam agents did, however, rapidly destroy foam which was formed by shaking tracheal edema fluid with air. These findings suggested that lung edema foam had uniques properties. Anti-foams, he noted, effectively destroyed such persistent foams as those formed by shaking

blood serum or detergent with air. The foam lavaged from animal lungs infused with unfoamed blood serum was resistant to anti-foaming agents. He also produced lung foam similar to that of acute edema in the absence of edema fluid. Sections of freshly excised normal lung squeezed under H_2O yielded bubbles forming a foam resistant to octyl alcohol and silicone anti-foam. *In vivo* and *in vitro* saline washes of lung produced similar foam. These observations suggested that some stabilizing properties associated with the lung foam came from the lung itself. He also discovered that lung bubbles washed gently with several changes of distilled water still retained their anti-foam resistance. Under the microscope, lung bubbles immersed in air-free water soon showed diffusion of air from the bubble interior to the water and visible "ghosts" of irregular form were all that remained. This suggested that the foam bubbles were lined with a solid substance which was permeable to air and insoluble in water. The peculiar properties of these bubbles were dependent upon this layer. Anti-foaming agents were effective in destroying the stability of the foam bubbles after they were incubated at $37^{\circ}C$. for four hours with proteolytic enzymes such as pancreatin or trypsin. Lung foam was also destroyed by a series of protein precipitants including ether vapor, mercuric chloride, amyl alcohol, and lower alcohols.

Use of anionic detergents, which displace many other substances such as saponin and albumen from liquid surfaces, did not affect the foam stability. These series of observations led Pattle (1958) to conclude that the layer lining lung foam bubbles was a highly surface-active, insoluble protein substance. He searched for other physiologic liquids that might have similar physical properties of the lung lining and form stable bubbles. Gastric mucin composed of mucopolysaccharides was the only substance which had similar physical properties in its ability to form stable foam. He noted, however, that the chemical staining characteristics of mucin differed greatly from the lung foam. On this basis, Pattle believed the lung bubble lining should not be classified as a mucoprotein. Pattle (1958) suggested that the stability of bubbles derived from the lung was due to low surface tension. To support his belief, he used the microscope to observe the contraction and decay of bubbles formed in various liquids which were in equilibrium with atmospheric pressure. He observed that bubbles expressed from lung and as small as 1μ in diameter were stable during a 25 minute observation period. Bubbles from sources other than lung and having high surface tension quickly disappeared because their individual internal pressures were greater than atmospheric pressure and as a result, gas rapidly diffused from the surface layer of the liquid to the bulk liquid surrounding the decaying bubble. A direct relationship existed between the

rate of gas passing to the bulk aerated liquid and the degree of bubble surface tension. Figure 2 shows that lung bubbles transferred to aerated water initially become slightly smaller and then remain stable for extended periods. He ruled out impermeability of the insoluble lining layer as a stability factor because of bubble decay in de-aerated water as described above. Using an equation derived by Epstein and Plesset (1950) for the lifetime of a bubble contracting slow enough to establish a steady state of diffusion from the bubble, and incorporating a correction factor (1/ln2 or 1.44) for the increased life of a bubble in a hanging drop slide preparation, Pattle calculated an upper limit of surface tension (γ) for lung bubbles equal to 0.026 dynes/cm. The modified Epstein and Plesset equation was written as: $T=(pr^3+2\gamma r^2)/(6D\lambda\gamma \ln 2)$ where: T=the lifetime of a bubble with radius r in a liquid having surface tension γ saturated with gas at atmospheric pressure p. D is the diffusion coefficient of the dissolved gas, and λ represents the ratio of the dissolved gas concentration to gas density in equilibrium with it.

In a different experiment, realizing that the ratio of bubble height in a liquid of known density is determined by the bubble's surface tension, he estimated the magnitude of the surface tension of bubbles expressed from lung to be less than 0.1 dynes/cm. He stated that "the cause of the stability of bubbles obtained from the lung is a true surface

film which exerts a surface pressure almost equal to the surface tension of the liquid in which they are immersed, or which, to put this another way, reduces the surface tension of the bubble almost to zero." (Prattle, 1958).

Prattle (1955) stressed the important role of pulmonary surface-active substances in preventing the alveoli from filling with blood capillary transudate. To emphasize that the surface tension at the air-alveolar interface was not identical to an ordinary air-liquid interface, he discussed some of Drinker's (1950) earlier work on the "dry lung." Drinker (1950) postulated that an equilibrium resulting from pressure balances existed between small pulmonary blood vessels and alveoli. Pattle (1965) stated that Drinker "ignored the possible effects of surface tension at the sharply curved alveolar surface." The factor opposing transudation is, according to Drinker, the osmotic pressure of the plasma proteins (35-42 mb) [millibar, unit of pressure, equal to 1,000 dynes/cm]; to this are opposed the pressure in the SBV [small blood vessels] (14 mb) and the net intrathoracic negative pressure due to breathing movement (7-14 mb). This leaves a balance of from 7 to 21 mb in favor of dryness. He took no account of the fact that in a hemispherical alveolus of radius 50 μ a surface tension of 55 dynes/cm [the value of blood serum found by duNouy (1926)] would produce a negative pressure around the small blood vessel of 22 mb, enough

to more than wipe out the balance in favor of dryness and replace it by a balance of 1 to 15 mb in favor of transudation."

Pattle (1966) also mentioned that: "By virtue of this tendency to contract a curved liquid surface exerts a pressure acting toward its concave side; for a spherical surface the pressure is given by $2\sigma/r$ as the lung has a large internal surface area (of order 300 cm^2/ml) and the alveoli have small radii of curvature (of order 50 μ), and furthermore are separated from the blood by a membrane which is far from impermeable, surface tension must be of high importance in the lung." The danger of transudation resulting from a tendency of alveolar surfaces to contract when surface tension is high is considered by Clements *et al*. (1958), Avery (1962), Mead (1961), and Macklin (1955) secondary to the problem of decreased terminal airway and alveolar stability and subsequent collapse in the face of high surface tension.

Regarding the common description of alveoli having sharply curved surfaces, it is important to digress momentarily at this point and consider the observations of Staub (1966) on surface tension and pulmonary circulation. In his studies on the interdependence of pulmonary structure and function, cat and human lungs were quick-frozen by inundating them with liquid propane cooled to -180°C. in liquid nitrogen. Segments of frozen lobes were fixed by freeze-substitution,

embedded in nitrocellulose, sectioned and then stained. This technique preserved the lung in approximately its state in life. Staub proposed a new morphologic model of the pulmonary alveoli. He suggested that the central portions of the alveolar walls were flat and only the wall junctions or corners were sharply curved. Surface tension forces, he suggested, act primarily at the curved junctions, creating an outwardly directed increase in the transmural pressures. This produces an enlargement of the alveolar capillaries at these sites. He presented a theoretical mechanism wherein surface tension acting on the curved junctions protects the pulmonary capillaries from the full effect of high intra-alveolar pressures. Therefore, although the capillaries in the flat walls compress with high alveolar pressures, flow is still maintained in the capillaries of the wall junctions. These findings are interesting in light of the "traditional" anatomic picture of sharply-curved alveoli and support Weibel's (1963) suggestion "an irregular polyhedral configuration."

The lining film, as mentioned above, has an important physiological function in maintaining the stability of the fine airways and air spaces. Normal elastic recoil of the lung is dependent upon the presence of elastic tissue and the surface active film lining the alveoli. The experiments of von Neergard (1929), described earlier in this paper, required less than half the pressure to inflate gas-free lungs

under static conditions with physiologic fluid than that pressure necessary to inflate air-filled lungs. The properties of the pulmonary elastic fibers were not altered by fluid filling. To account for the marked difference in the required inflation pressures, he concluded that each alveolus in mammalian lung presented a curved, air-liquid interface which retracted very similar to a bubble. The total recoil or retraction pressure of the vast number of alveoli present in the lung [about 300 million alveoli are present in the normal human lung (von Hayek, 1960, Weibel, 1963)] produces as much recoil pressure as that provided by all the elastic fibers. Fluid-filled alveoli do not have an air-fluid interface. These alveoli have a fluid-fluid interface with nearly no surface tension. "The recoil pressure of the fluid-filled lung measures the recoil pressure of only the elastic (or elastic-like) fibers. The recoil pressure of air-filled lungs measures the recoil of both the elastic fibers and the surface film." (Comroe, 1965a).

A soap bubble blown on a tube serves as a model for alveolar stability. The relevance of the model was very clearly explained by Mead (1960), Said (1965), and Comroe (1965b). Molecules in the interior of a homogenous liquid such as water have freedom of movement and experience equal intermolecular attracting forces in all directions. At the surface of the liquid, however, a water molecule is attracted with greater force by the large number of similar molecules

below and to the side of it than by the relatively few air molecules in the gaseous phase above it. This unequal attractive force acting on all the surface molecules tends to pull the surface downward and sideward. These attracting forces result in a surface tension (measured in dynes/cm) acting to shrink the surface to an area as small as possible. A soap bubble has a curved, air-liquid interface similar (discounting Staub's (1966) alveolar model for the moment!) to that of an alveolus. The driving force for bubble collapse is the surface (or interfacial) tension which creates an excess pressure as defined by Laplace's equation for a sphere: $P=\frac{2T}{r}$, where the pressure (P) in the bubble is related to twice the tension (T) in the wall of the bubble divided by the radius of the curvature (r). From this expression of Laplace, which also appears to be applicable for alveoli, surface forces will increase if the radius of curvature decreases or if wall tension increases (Said, 1965). Boys (1959) has shown that if two bubbles of radii R and r are connected by a flow tube, the larger bubble expands at the expense of the smaller one until the latter disappears into a flat film. On the basis of the model, the pulmonary alveoli which have different radii of curvature and are connected in series to a common airway present an inherently unstable system. Alveoli with small r have a high tension and would empty into larger alveoli resulting in overinflation and collapse of the terminal air sacs. It is apparent that

in normal lung, the alveoli can only be transiently unstable (Avery, 1962). At least two mechanisms are known to be important in promoting stability of the airways. Mead (1961) suggested that stability depended upon a positive compliance ($dv/dp>0$) and that its reciprocal dp/dv representing tissue elastance (or recoil) also had to be greater than zero. He stated that total elastance was the sum of the separate tissue and surface elastances. Stability of the air spaces was dependent, therefore, on the sum of its surface and tissue elastances being greater than zero. Brown et al. (1959) and Clements et al. (1961) were the first to respectively discover and provide experimental evidence that "surface elastance," the change in surface tension of the lung with area, was operating in the lung. Clements (1957) initially suggested this concept after he viewed the indirect evidence obtained from surface tension measurement on lung extracts (Clements, 1956). He also, with this concept, attempted to provide further understanding of the low surface tension in lung bubbles observed by Pattle (1958). Pattle's data were difficult to reconcile in light of the evidence, mentioned above, that the liquid-air interface contributed two-thirds to three-fourths of the lung elasticity at large volumes (Brown, 1957). On the basis of von Neergard's (1929) and Radford's (1954) measurements of high surface tension recoil for fully inflated lungs, Clements (1957) suspected that the surface tension of the alveolar lining film was much

lower at small lung volumes [expiration] in order to prevent alveolar collapse. He believed that this "surface elastance" must be peculiar to the alveolar lining film which contained a special type of surface-active substance. Other known physiologic fluids did not exhibit marked changes in surface tension with area.

STUDY ON THE BEHAVIOR OF AN ALVEOLAR LINING FILM

Clements' (1957) data were obtained from measurements of lung extracts by the method of Wilhelmy developed in 1863 which measured the downward pull of a surface layer on a stationary metallic bar. He modified this technique by placing saline extracted lung materials in a Langmuir-Adam trough containing saline. The extracts were obtained from small samples of lung tissue (1 to 3 gm) which were previously minced. These extracts were filtered and then poured onto the trough which permitted automatic compression and expansion of the surface area and simultaneous measurements of surface tension. Measurement of surface tension depended upon a downward force exerted by the fluid surface on a platinum strip 0.001 inches thick. The fluid surface presumably contained surface-active materials (surfactant) from the lung. This strip was suspended in the fluid from the arm of a sensitive force transducer. Surface area of the fluid was varied cyclically by a motor-driven barrier in contact with the surface. In a solution containing surface-active

and non-surface-active molecules, the former molecules are attracted less strongly than the latter and collect at the surface. Since these surface-active substances (or surfactants) concentrate at the surface and have inherently small attractive forces for other kinds of molecules present in the bulk phase, they effectively dilute these molecules and therefore lower the solution's surface tension. Clements' (1957) in vitro surface tension measurements of lung extracts varied from 2 to 5 dynes/cm during surface film compression to 40 to 50 dynes/cm on expansion. Clements et al. (1961), using their own findings and those of others mentioned previously, proposed a mathematical model of the lung and calculated how it might behave under conditions where surface tension would cause alveolar collapse. They concluded that alveolar stability was favored by high internal pressure, a large radius, low surface tension and high surface elastance. The surface tension of the alveolar lining approached 40 to 50 dynes/cm when the alveoli were large and 2 to 5 dynes/cm when they were small. Thus, the film itself was elastic and demonstrated hysteresis.

As mentioned earlier in this paper, Pattle (1958) measured surface tension less than 0.1 dynes/cm in his lung bubble studies. Avery and Said (1965) pointed out the surface tension value for a bubble expressed from the lung was lower by comparison to the values obtained during surface compression in the modified Wilhelmy apparatus. They

reconciled this difference by suggesting that the surface film in a contracting bubble was highly compressed and therefore its surface tension was comparable to that measured on the Wilhelmy balance during surface area reduction.

An increasing number of studies on actual lung have provided support for the theoretical models described by Pattle (1958) and the research group of Clements (1957). Clements (1960) has given more direct evidence for his model. He found a high degree of correlation between the retention of air in lungs deflated to low pressures (5 cm H_2O) and the large variations in surface tension of extracts from these lungs. Excised lungs of infants who have died of the respiratory distress syndrome: showed high minimum surface tension of saline extracts and narrow hysteresis loops, required high inflating pressures, collapsed rapidly at low inflating pressures (Avery, 1964) and produced unstable bubbles (Pattle, et al. 1962). Animals chronically exposed to cigarette smoke (Giammona, 1967a) and sulfur dioxide (Kahana and Aronovitch, 1966) showed decreased maximum surface tension in extracts of lungs and in volume pressure determinations. Endotracheally administered furniture polish (a frequent type of petroleum product intoxication in children) in rats and dogs produced a significant increase in the minimum surface tension of pulmonary extracts (Giammona, 1967b). An atelectatic lung will remain inflated for a long duration and retain its normal pale pink color (Pattle, 1958). However, if

surfactant is deficient as in the respiratory distress syndrome or is contaminated by detergent solutions instilled into the lung (Radford, 1963) collapse occurs immediately after inflation and lung bubbles show abnormal stability (Pattle and Burgess, 1961). Lungs and saline extracts of lungs heated to 47°C. produced atelectasis at normal end-expiratory transpulmonary pressures and loss of ability to reduce surface tension to very low values (Clements, et al. 1961). These experiments reflect only a part of the "massive body of evidence to show that the lining film and lining complex [to be described later in this review], by lowering the minimum surface tension and providing for a rapid variation of tension with area, acts so as to enable the lung to retain air at low inflation pressures." (Pattle, 1965).

USEFULNESS AND LIMITATIONS OF MAJOR TECHNIQUES USED IN PULMONARY SURFACE TENSION STUDIES

Static Pressure-Volume Measurements on Excised Lungs

Changes in pressure-volume characteristics, e.g., high opening pressures and narrow hysteresis loops in degassed lungs of infants who have died of the respiratory distress syndrome (RDS); (Gribetz et al., 1959, Gruenwald et al., 1962), can be compared to surface tension measurements of extracted lining film. Clements et al. (1961) established an expansion index relating the lung volume on deflation to

5 cm H_2O internal pressure as a fraction of the maximum volume.

Reproducible results of pressure-volume curves depend on use of a whole lung. Pattle (1965) pointed out the difficulties inherent in quantitative measurements of air retained in segments of inflated lung. He also suggested the possibility of experimental error due to difficulties such as interference from exudative material, leaks, and foam formation.

Studies of the Lining Film with the Modified Wilhelmy Balance

Avery and Said (1965) stated that the "dynamic studies of surface area-surface tension relationships all show the material expressed from lungs has a remarkable property of achieving a very low surface tension at reduced area, and a high surface tension at expanded area. This is to say the film itself is elastic and demonstrates hysteresis. The minimum surface tension that can be achieved depends in part on the concentration of the surface-active molecules in the surface film. There is a lower limiting tension, which may occur after a small reduction in area if an abundance of surface-active molecules are present, or after a larger reduction in area if only a small concentration of material is present. Surface tension as measured will fall as the density of surface-active molecules increases. Presumably when a

lower limiting tension is achieved, film collapse has occurred and no further increase in density can occur.

Clements (1965) concluded that extract surface tension depended upon the quality and the concentration of surfactant present. He suggested, therefore, that the variation in alveolar stability [low minimum surface tension and large hysteresis loops in normal lung] may be the result of similar changes in the surfactant at alveolar surfaces. He and his associates (Clements et al., 1961, Gruenwald et al., 1962) proposed an extract stability index $\bar{S}$, defined as the ratio of the difference between maximum and minimum surface tensions to the mean of those values. A theoretical maximum $\bar{S}$ of 2 was porposed. A range from 0.80 to 1.82 was found for normal lung, and values less than 0.2 for rat lungs altered by instilled detergent.

There are a number of variables inherent in current lining film studies. These include: difficulty in quantitatively determining surface film concentrations and dust contamination of the film (Reiss, 1968); some hysteresis in any mechanical system involving friction between parts such as the friction of the stylus and paper in continuous surface tension recording as surface area is changed (Greenfield and Kimmell, 1967); contamination impurities of extracts and washings (Mendenhall, 1963); marked differences in the rate of film compression-expansion cycle as compared to a normal cycle of breathing in some experiments (Mendenhall and

Mendenhall, 1963); errors in the estimates of surface tension due to the buoyancy produced by vertical movements of the balance arm in all Wilhelmy-type surface balances (Mendenhall, 1967); the completeness of extraction and the extraction per gram of lung tissue also are possible sources of error in evaluation of data (Levine and Johnson, 1957, Yeh et al., 1966).

Stability Measurements of Bubbles Expressed from the Lung

These methods were first developed by Pattle (1958). He air-inflated small segments of lung with a hypodermic needle. The aerated fragments about 1 mm in diameter were then squeezed with a forceps and the bubbles expressed were transferred into a drop of water hanging under a microscope slide. A hanging drop preparation was made by placing the slide over a depression slide. He observed the bubbles through a microscope fitted with an eyepiece graticule and measured bubbles having initial diameters ranging from 35 μ to 60 μ. Bubbles with diameters below 35 μ contracted too rapidly and those larger than 60 μ contracted too slowly for convenient calculations of a bubble stability ratio. This ratio provides an index enabling an investigator to compare behavior of bubbles derived from different sources. Pattle (1958) defines his concept of the stability ratio (sr) as "the inverse of the ratio of the area of the surface (or surface area of the bubble) to that which it must attain in

order for the surface tension to be reduced to nearly zero. For instance, if a spherical bubble of diameter d_1 contracts and remains stable at a diameter d_2, its stability ratio before contraction will be given by d_2^2/d_1^2." He noted that stability ratio values for normal lung ranged between 0.60 and 0.87, with a mean of 0.71. Values lower than 0.60 in his studies denoted a deficiency of lining film. Stability ratios above 0.87 were found in edematous lungs. It was also stated that the mean stability ratio determined by other investigators might vary with technique. Pattle (1958) presented convincing arguments based on his experimental evidence that the film lining the bubbles was originally the alveolar surface lining. He found that the typical foam associated with acute lung edema could be produced in rabbits whose respiratory and body movements were arrested by urethone asethesia and by injections of novocain into the cervical region of the spinal cord. Oxygen mixed with ammonia gas (to produce edema) was introduced into the trachea. At autopsy, foam present in the bronchi was resistant to treatment with anti-foams. Liquid which was also found in the airways was agitated with air and the foam produced was destroyed by silicone anti-foam and octyl alcohol. Pattle (1966), in reviewing this work, suggested that the resistant bubbles' origin was not from "large masses of air broken up and mixed with edema fluid by respiratory movement, and must therefore have come from a situation where there was a body

of air having a surface area large in relation to its volume. This situation could only have been the fine air spaces of the lung. The evidence suggests that the edema-foam was formed by the air in these spaces being trapped, broken up into bubbles, and then expelled into the bronchi. That the lining film of the bubbles must consist of the original lining film of the alveoli may be proved as follows. The surface film of the bubbles cannot have come from the edema fluid, because foam formed from such fluid has entirely different properties. Indeed, if lung foam is produced by injecting saline down the trachea, the liquid draining from it can hardly form a foam at all. Bronchial mucus does not form stable bubbles, so that the lining film cannot have been derived from the bronchial tree. The film must therefore have been acquired in a situation where the air was last in contact with a solid body, and must have been derived from the surface of such a body. This body can only have been the alveolar wall. The smallest bronchioles which were examined do not seem to give rise to stable bubbles, and it seems that the capacity to form the film is present only below the termination of the bronchial epithelium."

Niden (1967), whose work will be discussed below, presents evidence that the site of film formation is not necessarily restricted to regions below the terminal bronchioles.

Pattle's bubble method enables one to easily investigate localized and multiple samples of lung tissue. Since the film lining the bubble is presumably the original surface lining, stability ratios should reflect the *in vivo* state of the lining.

Some of the errors and limitations in measurements may arise from: blood contamination (Taylor and Abrams, 1964); introduction of extraneous bubbles (Pattle, 1965); difficulty in estimating surface activity of the bubble lining layer during expansion (Mead, 1961); enlargement of bubbles due to heat from light source used in observations (Slavokovic and Ellison, 1967).

In all the methods mentioned, inconsistencies in the literature may arise because some investigators interpret their surfactant studies as purely quantitative even though extraction efficiency is unknown (Clements, 1965). The quantitative determination of surfactant is also not realistic because its exact chemical composition has not, as yet, been resolved (Sekulic *et al.*, 1968a).

Post-mortem and storage effects on surfactant have been studied. Pattle (1965) mentioned such changes were irregular but may be rapid. Lung tissue kept at 37°C. for 24 hours usually lost its capacity for forming a lining film. Howatt and Strang (1965) found storage of fetal lamb lung tissue by freezing at -15°C. for 112 days did not alter surface tension measurements obtained by the Pattle and modified

Wilhelmy methods. They also noted that both methods showed statistically significant positive correlation of surface tension determinations. Normal infant lungs refrigerated at 0 to 4°C. for six weeks showed no change in subsequent surface tension measurements (Gruenwald et al., 1962). Gruenwald et al. (1962), Pattle (1965), and Slavkovic et al. (1968) found significant losses in surface activity after freezing pulmonary tissue for two months at 0 to 4°C. Slavkovic et al. (1968) noted that incubation of lung samples for two to four hours after death produced a significant decrease in capacity to form a lining. They suggested, however, that the decrease was probably negligible and not prohibitive for evaluation of results until six hours had elapsed. Sekulic et al. (1968b) made a direct statistical comparison of the Pattle and modified Wilhelmy methods on normal and atelectatic lungs of adult dogs. Extracts used in the Wilhelmy trough were prepared by mincing and homogenization of lung samples and by airway lavage. They found that Pattle's method and surface tension measurements in minced and homogenized lung extracts provided values which were most consistent and showed the highest correlation. Airway washing results had a lower correlation with those of the other methods. Slavkovic et al. (1968) suggested Pattle's bubble-stability method was sensitive enough to differentiate between gradations of normal.

From all the major methods used to determine if a lung sample has the capacity of forming a normal lining, a unifying physiologic concept emerges. The function of surfactant "is to keep the alveoli open and free from transudate by lowering the surface tension." (Pattle, 1966).

PHYSICAL AND CHEMICAL CHARACTERISTICS OF THE ALVEOLAR LINING LAYER

Macklin (1954) was the first to suggest that the interface between alveolar air and lung tissue was lined with a secretion from specialized alveolar epithelial cells which he called "granular pneumonocytes." From observations on stained, fresh and fixed lung tissue, he believed this lining to be a hydrated secretion containing acid mucopolysaccharides and myelinogens. He also suspected that the film was important in maintaining a "constant favourable alveolar tension."

Pattle and Thomas (1961), Klaus et al. (1961), Buckingham (1961), and Abrams and Taylor (1964) described the lipoprotein characteristics of extracted surfactant.

Clements (1967a) postulated the functional significance of protein associated with lipids in lung extracts and saline washings. He noted that many of the lipids in the extracted complex did not satisfy the criteria necessary to maintain low surface tension for prolonged intervals. These requirements [as determined from analysis of well-known

extra-pulmonary surface-active agents] included strongly polar functional groups at one end of the molecule and two long hydrocarbon chains at the other end. Another prerequisite was a lack of ethylenic linkages in the cis-configuration. In spite of these shortcomings, the lipid-protein complex was strongly surface-active. He suggested that the protein as a subfilm had an ordering influence promoting the stability of "a mixture of lipids which by itself would not long maintain an orderly monolayer at low surface tensions (that is, at high film concentrations)." He proposed that the lipoprotein layer was a duplex with lipids comprising the top layer and protein the layer below.

Based on electron micrographs of the superficial, osmiophilic, extra-cellular layer over the alveolar surface, the actual surface layer's thickness has been estimated to be about: 40 Å (Weibel, 1967), 50 Å (Pattle, 1967a), and 50 to 60 Å (Clements, 1967b).

Pattle (1967b) postulated that a layer of insoluble lipoprotein about 50 Å thick existed immediately next to the alveolar air and could be detached and floated off as a bubble lining. "Below this is a layer of jelly or slime whose thickness is unknown; it may be about 500 Å or 2,000 Å thick. This layer is of lipoprotein composition and can be dispersed in saline to give a liquid with peculiar surface properties. When the surface area is increased, as during a deep breath, matter from this layer can be adsorbed onto the

surface and become part of the insoluble lining film, the surfactant layer is apparently mechanically weak and should not be referred to as a membrane. Below it are the anatomical structures of the blood-air barrier, whose thicknesses, as revealed by electron microscopy [for human lung], are given by Schultz (1962) as the extended processes of the pulmonary epithelial cells (400 to 650 Å); the basement membrane (1,100 to 1,600 Å); and the endothelium of the capillaries (200 to 4,000 Å)." Pattle (1966) also suggested that the complex had a very high molecular weight. If this were not so, the osmotic balance between the alveoli and the blood would be upset.

Clements (1967c) pointed out that surfactant differed in several ways from other agents that lower surface tension, such as bile salts and detergents: (a) When a film of surfactant is compressed, it stays on the surface in high concentration and with a very low surface tension; (b) similar treatments to other surface-active agents results in a movement of the agents from the surface phase into the bulk phase. (Thus, lung surfactant is, inappropriately, called insoluble and other surface-active materials are referred to as soluble.)

Chase (1959) used the PAS method on freeze-dried lung tissue and electron microscopy to observe the alveolar surface. He found a PAS-positive acellular layer covering the alveolar epithelium in the bat, rat, mouse and chicken.

Tyler and Pangborn (1964) noted a laminated membrane on electron micrographs of the epithelial surface of chicken lung tertiary bronchi and atria fixed with buffered osmium tetroxide. These workers also observed, by light microscopy, tissue sections one micron thick stained with the periodic-acid-Schiff (PAS) method, hematoxylin and eosin, and toluidine blue. They suggested that the surface lining was necessary for the surface tension-reducing properties of avian lung extracts.

Kikkawa et al. (1965) were the first to demonstrate with electron microscopy, an osmiophilic acellular layer lining the alveolar surface of mammalian lung in situ. Their methods included use of lamb lung fixed in cold, Veronal buffered, one per cent osmium tetroxide. Klika and Janout (1967) also reported in situ visualization of an osmiophilic lining-complex on the alveolar surface. They examined electron micrographs of rabbit lung fixed and stained with Maillet's modification of Champy's method. This technique includes a 1:3 mixture of one per cent osmium tetroxide and zinc iodide.

Groniowski and Biczyskowa (1964) stained lung tissue with Hale's reagent for mucopolysaccharides. With electron microscopic studies, they found a Hale-positive continuous coat over the alveolar epithelial lining. The thickness of the layer was approximately 300 Å. They suggested that the

surfactant complex layer consisted of mucopolysaccharides and lipoprotein.

Positive osmiophilia indicated phospholipid or lipoprotein components of the alveolar lining layer (Tyler and Pangborn, 1964). The mucopolysaccharide layer observed by Groniowski and Biczyskowa (1964) was not known to be osmiophilic or to possess surface activity and thus needed to be investigated further (Kikkawa et al., 1965).

Clutario et al. (1966) and Scarpelli et al. (1967) analyzed saline tracheal lavages and extracts obtained by mincing lung tissue previously perfused via the pulmonary circulation. They also analyzed mammalian serum in parallel studies. These workers found the surfactant system of the lungs to be a complex mixture of lipids and polysaccharides. The lipid fractions contained the phospholipids lecithin, phosphatidyl ethanolamine, sphingomyelin, and lysolecithin in decreasing order of concentration. Other fractions contained neutral lipids and high concentrations of complex polysaccharides. Little or no protein was evident in the system. There was a high concentration of protein and a "potent surfactant" in the blood serum. Because of these results, Scarpelli (1968) took issue with the above descriptions of a surfactant lipoprotein complex. He stated that the lipoprotein found in lung extracts was a contamination of blood. These studies indicated that a phospholipopolysaccharide complex was lung surfactant or an essential component of it.

Weibel and Gil (1968) pointed out that results which suggested a lining rich in mucopolysaccharides coincided with Brandt's (1962) observations of extraneous mucopolysaccharide coats on many "free" cell surfaces.

A careful review of the literature on this issue ("protein or not protein") revealed that a great majority of investigators supported the existence of a protein moiety in the surface film lining. The following list includes only some of these workers: Clements (1967a); Kikkawa et al. (1965); Campiche (1960); Mendenhall and Sun (1964), Tyler and Pangborn (1964), Bolande and Klaus (1964), Felts (1965), Galdston et al. (1965), Klaus et al. (1962), Taylor and Abrams (1966), Pattle (1966), Said (1967), and Weibel et al. (1966).

Of the papers, mentioned above, which reported in situ visualizations of a lining at the alveolar-air interface, none successfully demonstrated its distinct separation from the epithelial cell membrane. They also failed to show direct evidence of the lining's protein and lipid components (Weibel and Gil, 1968). In a very interesting paper, Weibel and Gil (1968) clearly demonstrated an alveolar lining layer which was independent of the external plasma membrane of the alveolar epithelial cell and had two distinct phases. One of the phases consisted of polar lipids.

Weibel and Gil (1968) suggested that previous failures clearly demonstrate the two parts of the extracellular

lining layer were due to inadequate fixation techniques. They mentioned that the freeze-drying method of Chase (1959) had limitations. The other techniques employed were unsuccessful because alveolar air was substituted by an aqueous solution. They stated that the hydrophobic properties of the surfactant were incompatible with a change from air to water. Thus, an aqueous solution instilled via the airways would destroy and wash off the surfactant lining unless it had previously been fixed by diffusion from capillaries to the alveolar surface.

A technique developed by Forssmann et al. (1967) to perfuse and fix tissue via the vascular bed was modified by Weibel and Gil (1968) for use on rat lungs. His subsequent electron micrographs clearly demonstrated that the extracellular lining layer had two main phases: (1) a base layer containing an aqueous solution of proteins, mucopolysaccharides and lipids; (2) a lamellar superficial layer of polar lipids and water.

These observations supported the "composition of surfactant postulated from chemical data" presented by Pattle and Thomas (1961) and Klaus et al. (1961).

SURFACTANT SYNTHESIS IN THE LUNG

Klaus et al. (1961) chemically analyzed dried foam from beef lungs and found that 74% of the lipid fraction was comprised of phospholipids. These workers also reported

that dipalmitoyl lecithin was the predominant phospholipid in the lipoprotein foam. They also demonstrated that purified samples of dipalmitoyl lecithin had surface activity similar to that of crude lung extracts. Brown (1964) and Morgan et al. (1965) also found high concentrations of dipalmitoyl lecithin in their analyses of lung extracts.

Comroe (1965b) pointed out that the lung in comparison to other mammalian organs contained large quantities of saturated fatty acids, especially palmitic acid. Adult lung tissue in vitro had a high rate of uptake of palmitic acid. The phospholipids of the lungs were rich in saturated fatty acids, and the ratio of saturated to unsaturated fatty acids in this group was exceptionally high. Saturated phospholipids gave the lowest values of surface tension and the most stable surface films. He suggested that their synthesis may be the purpose of lipid metabolism in the lungs. Late in fetal life, when surface activity appeared, the quantity of saturated phospholipid increased and the ratio of saturated to unsaturated phospholipid tended to rise.

Harlan et al. (1966) examined the metabolism of palmitoyl lecithin in normal and experimentally induced edematous dogs after intravenous injection of radiolabled (C^{14} and H^{3}) free palmitic and oleic acids. In normal dogs, incorporation of palmitic acid into pulmonary phospholipids was four times more rapid than that of oleic acid, and primarily occurred in lecithin. There was a significant decrease

in total phospholipid, phospholipid radioactivity, and surface activity in the edematous animals. (Pulmonary edema was induced by intravenous infusion of dextran.) Similar results were also obtained for atelectotic portions of edematous lung when compared to aerated areas. The observations substantiated the importance of surface-active phospholipids, primarily dipalmitoyl lecithin, in preventing alveolar collapse. These investigators also noted that the phospholipid radioactivity in liver and lung was similar and that the activity in plasma was less than either. They concluded that pulmonary phospholipid synthesis occurred within the lung.

Chida et al. (1966) demonstrated qualitative and quantitative changes in lipids of fetal lamb lung extracts during the last weeks of gestation. These changes were related to differences in surface activity. Their results showed that the milligrams of lipids per 100 ml of fetal lamb lung extract increased with body weight or gestational age. The percentage of palmitic acid in lecithin also increased. This suggested a probable increase in dipalmitoyl lecithin. The stability index (Clements et al., 1961) of surface films measured on a modified Wilhelmy balance increased toward term. In vitro studies by Chida and Adams (1967) on acetate -1-^{14}C incorporation into phospholipids by lung slices from fetal and newborn lambs and ewes strengthened the hypothesis of de novo synthesis. These workers determined the fetal lung's ability to synthesize long-chain fatty acids

and lecithin from an acetate precursor. They also investigated maturational differences in lecithin synthesis. Their results indicated that the lung incorporated the labeled precursor into myriatic, palmitic and C_{18} fatty acids. Myriatic and palmitic acids were the predominant radioactive saturated lecithin fatty acids. After examining the distribution of label, they suggested that the majority of acetate incorporated into palmitic acid occurred through total synthesis via malonyl-coenzyme A. Only small amounts of acetate were involved in the elongation of 16:0 to C_{18} fatty acids by addition to preexisting acyl groups. They also noted a marked increase of acetate incorporation in phospholipids, "especially lecithin," during maturation. They related this observation to saturated lecithin's importance, as a component of lung surfactant, in the aeration process at birth.

Their results differ somewhat from those of Scarpelli (1968) in regard to the importance of phosphatidyl ethanolamine transmethylation as a pathway for lecithin synthesis. A direct transfer of methyl groups from methionine to phosphatidylethanolamine is known to be a pathway for lecithin (phosphatidylcholine) synthesis in liver (Ansell and Hawthorne, 1964). Chida and Adams (1967) stated that their results indicated transmethylation was not as important as other possible pathways of lecithin synthesis, such as the condensation of diglyceride with phosphorylcholine.

Scarpelli (1968) injected C^{14} labeled Na-palmitate into adult rabbits and sacrificed the animals after five minutes, thirty minutes, four to six hours, and fifteen hours. Corresponding lung tissue and washings were examined for radioactive lipid content by silica-gel thin-layer chromatography. Within five minutes, the label was evident in washings and at highest concentrations in the phospholipids associated with migrated phosphatidyl ethanolamine. A relatively small amount of C^{14} label was found in the lecithin fraction of the chromatogram. Radioactive label was also present in lung parenchyma extracts. Specific activity of lecithin was relatively higher here than in washings. "During the next 15 hours there was a progressive fall in the phosphatidyl ethanolamine activity and a net rise in lecithin activity, so that by four to six hours the phosphatidyl ethanolamine: lecithin activity ratio was reversed. At 15 hours there was no phosphatidyl ethanolamine activity in the washed lung [parenchyma] and little lecithin activity, whereas activity was present in both phospholipids of the lung washings. These studies suggested that (1) the lung synthesizes phospholipids and presents them to the lining layer extremely rapidly; (2) the phosphatidyl ethanolamine of the lining layer is converted to surface-active lecithin [via transmethylation]; (3) this process may take place within the lining layer itself; and (4) lecithin remains in the lining for a longer period of time than it does in the lung parenchyma."

Gluck et al. (1967a) reported that lecithin was the most abundant of the pulmonary phospholipids in developing and newborn rabbit lung and showed characteristic changes that correlated closely with lung surface activity. However, not all lecithins detected were surface active. They also demonstrated surface activity in purified lung phospholipids occurring in sphingomyelin, dimethylphosphatidyl ethanolamine and phosphatidyl inositol. Their individual surface activities did not correlate as well with lung as did lecithin. Surface-active lecithin appeared in fetal lung parenchyma long before it was observed in alveolar washes and comprised approximately eleven per cent of total alveolar lecithins in the term fetus. One hour after the onset of breathing this increased dramatically to adult levels of about 50% in the term newborn rabbit.

After extensive in vitro and in vivo experimentation on the biosynthesis of phospholipids in lungs of fetal and full-term breathing rabbits, Gluck et al. (1967b) determined that the major pathways for the de novo biosynthesis of lecithin in fetal lung were: (1) the incorporation of CDP-choline+D-α,β-diglyceride; and (2) the methylation of phosphatidylethanolamine. "The principal pathway for the de novo synthesis of phosphatidylethanolamine was found to be incorporation of CDP-ethanolamine+D-α,β-diglyceride and was the most active of the fetal pathway studied. During fetal development there was a progressive decline in activity of

CDP-choline incorporation. The methylation reaction had peak incorporation coinciding with viability of the rabbit fetus. However, with the onset of breathing, 90% of the de novo synthesis of surface-active alveolar wash lecithin was by incorporation of choline. The methylation reaction appeared to be of significance in the alveolar layer metabolism of the rabbit after some days of life and thereafter when the surface-active intermediate, phosphatidyl dimethylethanolamine, was recovered from alveolar wash. Methylation enzymatic activity was present in cell-free alveolar wash of adult rabbit."

They also noted that incorporation of labeled methyl groups (via radioactive S-adenosyl-L-methionine) was complete by 15 min. in lungs of 28-day rabbit fetuses. However, in adult animal tissue, the transmethylation continued during an entire hour of observation. It was suggested, assuming valid differences in reaction rates based on equal access to available endogenous substrates, that this might account for the paucity of measurable intermediates, phosphatidyl methyl and dimethanolamine in parenchyma and alveolar wash of fetal lung.

In a report subsequent to the one above, Gluck et al. (1967c) indicated that after simultaneous intraperitoneal injections of ^{3}H-choline and ($^{14}CH_3$)-methionine into immature rabbits delivered by Caesarean section rapid synthesis of lecithin occurred by the incorporation of CDP-choline and

D-α,β-diglyceride. Small, premature human infants differed markedly in that they were more dependent upon the pH sensitive pathway of triple transmethylation (phosphatidyl ethanolamine (PE)+$CH_3 \rightarrow$ P-methyl-E+$CH_3 \rightarrow$ P-dimethyl-E+$CH_3 \rightarrow$ lecithin). Human tracheal aspirates were studied using the parenterally injected surface-active intermediary phosphatidyl dimethylethanolamine as a radioactive marker.

Chida and Adams (1967) studied the in vitro relation between fetal maturation and the rate of incorporation of ^{14}C labelled palmitate, glucose, and choline into the phospholipids found in lamb lung. They noted an increase in the rate of incorporation of the above precursors, especially lecithin, as the fetus matured. Palmitate incorporation after birth was less active in the liver than in lung for one month. By three months after birth, liver activity was greater. It was suggested that the maturing fetal lung was uniquely prepared for phospholipid synthesis and lecithin in particular. They also proposed that the enzyme systems involved in phospholipid metabolism mature at different rates in different organs. Inadequate development of enzyme systems and subsequent inactive synthesis of lecithin was discussed as a possible cause of the respiratory distress system (RDS) which is usually accompanied by deficient surfactant.

Adequate pulmonary blood flow appears to be necessary for the synthesis of qualitatively and quantitatively

sufficient surfactant. Naimark (1966) observed incorporation of palmitate -1-^{14}C into lung tissue lipids after experimentally induced acute variations in the pulmonary blood flow of mongrel dogs. The effects on flow by left pulmonary artery ligation, left main bronchus ligation atelectasis and the influence of gravity (using the X_e^{133} technique) were studied.

Pulmonary artery ligation produced a marked reduction in the incorporation of labelled palmitate into both neutral and phospholipid fractions. This indicated that the bronchial circulation contribution to fatty acid uptake was negligible (at least in experiments of an acute nature). In atelectatic lungs, palmitate incorporation into phospholipid was decreased in proportion to the decrease in blood flow. The uppermost regions of lung in upright dogs were relatively poorly perfused and labelled fatty acid uptake was slightly lower than in lower regions. Supine dogs did not show these differences.

Finley et al. (1964) demonstrated deficiencies in normal surfactant production following unilateral pulmonary artery ligation. There was an increase in surface tension of lung extracts within hours after the ligation. They were able to correlate the development of atelectasis with abnormal surfactant. Extracts of the contralateral, well-perfused lung had normal surface activity.

The return of normal lung function and surface activity after six months was postulated as a progressive increase in perfusion by development of a bronchial artery collateral circulation.

Similar alterations after PA ligation were observed by Greenfield and Kimmell (1967).

Sekulic et al. (1968b) in experiments on dogs estimated effective bronchial artery collateral flow after left pulmonary artery ligation. They also observed pulmonary artery (PA) ligation effects on lung bubble stability and extract surface activity.

Complete atelectasis of left lung was evident on the fourth day following left PA ligation. Increased surface tension was indicated by low bubble stability ratios and high minimal surface tension in compressed extract films. After 12 days atelectasis diminished, and at 30 days was no longer present.

Bronchial artery collateral circulation increased from less than 5% of the total cardiac output on day one post-PA ligation to 22% after one month. This indicated that collateral circulation developing from the bronchial system was sufficient for adequate surfactant synthesis.

Clements (1963) postulated a sequence of events that might result with inadequate pulmonary perfusion. Metabolism of cells in the alveolar region would be improperly maintained and synthesis depending upon oxidative metabolism

would be disrupted. This would also include interference with cellular barriers and transport and secretory mechanisms. These alterations would result in deficiency of surfactant, leading to reduced pulmonary compliance and subsequent insufficient maintenance of ventilation. In turn, decreased ventilation would interfere with alveolar perfusion, thus completing a vicious cycle.

A CLARIFICATION OF ALVEOLAR EPITHELIAL CELL NOMENCLATURE

Before reviewing evidence in the literature for specific cells as sites of surfactant synthesis and secretion, it is deemed necessary to discuss current thought concerning alveolar epithelial cell types.

Miller (1932) was one of the first workers known to express belief in an alveolar surface epithelium. The electron microscopic studies of Low (1952, 1953) established the existence of a true respiratory epithelium lining the pulmonary alveolar walls. He observed that this cell lining faced the air spaces and cytoplasm attenuated abruptly at the nuclear margins to form a thin sheet of cytoplasm resting on a basement membrane.

Bertalanffy (1965) recommended that Low's designation of "pulmonary epithelium" or its modified form "pulmonary surface epithelium" be adopted as part of a proposed uniform nomenclature for the cells of respiratory tissue. He also suggested that the term "alveolar cell" be used to describe

what he believes is a somewhat pleomorphic cell with phagocytic and ameboid potential found in, or on, the alveolar wall.

Brooks (1966), Policard et al. (1957), Suzuki (1966a), and Sorokin (1967), to name a few, presented electron microscopic evidence that permitted a more precise definition of cell morphology and thus a more accurate basis for application of appropriate cell terminology. These ultrastructural studies have shown two morphologically different alveolar epithelial cell types. One type is the squamous alveolar cell described by Low (1952, 1953). The literature has an abundant supply of confusing terms for these cells, e.g.: small alveolar cells; type I cells; type A cells; membranous pneumonocytes.

The other cell type described by Bertalanffy has also been given a surplus of names, e.g.: alveolar cells, granular pneumonocytes, septal cells, corner cells, niche cells, type II cells, type B cells, large alveolar cells. This cell, according to Brooks (1966), "is, at the electron microscope level, not a pleomorphic, phagocytic cell, but rather an epithelial cell having a definite and relatively invariant morphology." Sorokin (1967) has named these, "great alveolar cells." He noted that, unlike the attenuated epithelial cells (type I), these cells had their vacuolated cytoplasm concentrated around a large, vesicular nucleus. This gave the cells an irregular cuboidal shape and enough

thickness for light microscope observations. Its ultrastructure demonstrated characteristic inclusions termed cytosomes or multilamellar bodies. He disagreed with Brooks (1966) regarding the cell's pleomorphism and suggested this occurs to a large extent within limits described as cuboidal. Sorokin described several characteristics which support the epithelial nature of the great alveolar cell. These are as follows: "(1) The cell rests directly on the basal lamina of the alveolar epithelium. (2) It exhibits polarity in that its generally apical free surface is endowed with microvilli. (3) It forms junctional complexes with processes from squamous alveolar epithelial cells. (4) In the fetal lung, moreover, inclusions similar to the cytosomes of great alveolar cells are found only in cuboidal epithelial cells that line distal ramifications of the bronchial tree, where formation of alveoli is destined to occur. Such cytosomes are not found in the maturing muscle or connective tissue of the lung. In being present in both the primitive respiratory epithelium and the great alveolar cells, the cytosomes argue both for the epithelial nature of the great alveolar cell and for its derivation from the primitive cuboidal epithelium." Because of its thickness, Sorokin (1967) suggested that the great alveolar cell was not involved in gaseous exchange.

Most of the recent papers on the lung recognize the epithelial nature of this cell and refer to it as a type II

cell after Campiche (1960) or as a great alveolar cell after Sorokin (1967).

Bertalanffy (1964) indicated that the great alveolar cell gave rise to the free alveolar phagocyte (wandering macrophage, dust cell, pulmonary phagocytes, or type III cells). However, Oren et al. (1963) and Dannenberg et al. (1963) noted that during phagocytosis, the alveolar macrophage was metabolically distinct from both the mesodermal polymorphonuclear and monoculear phagocytes. Other differences were noted by Low and Sampaio (1957) and Karrer (1958) who challenged the intact alveolar epithelium with Thorotrast and India ink and found considerable uptake of the materials by the phagocytes but little or none by the intact epithelial cells. In a later study, Ladman and Finley (1956) observed ingestion of Thorotrast by great alveolar cells isolated from dog lung. Krahl (1964) described alveolar epithelial cell ingestion of tracheally instilled carmine or carbon particles.

Conflicting reports have occurred regarding the activity of the hydrolytic enzyme, acid phosphatase. No activity in great alveolar cells and high activity in alveolar phagocytes was observed by Buckingham et al. (1964). Some acid phosphatase activity in great alveolar cells was noted by Balis and Conen (1964), Sorokin (1967), and Garcia and Valdiva (1967).

Whether or not the free alveolar macrophages are transformed great alveolar cells that become detached from the epithelial lining (Greep, 1966) remains to be resolved.

SITE(S) OF SURFACTANT SYNTHESIS AND SECRETION

Evidence for the Great Alveolar Cell

Macklin (1954) was the first to suggest that the extracellular alveolar lining was derived from "granular pneumonocytes" (great alveolar cells). With light microscopy he observed cytoplasmic, osmiophilic granules which were eventually "discharged" at the air-alveolar interface. On the basis of electron microscope studies, these granules have since been described as dense, laminated inclusion bodies (Low, 1954, Karrer, 1956, Policard et al., 1957, Woodside and Dalton, 1958, Campiche, 1960, Balis and Conen, 1964, Amirana et al., 1964, Sorokin, 1967, Weibel and Gil, 1968). Balis and Conen (1964) noted that their electron opaque appearance was generally similar to the myelin figures described by Revel et al. (1958). Bensch et al. (1964), Balis and Conen (1964), and Kikkawa et al. (1965) found inclusion bodies limited by a unit membrane which, at times, was continuous with deep infoldings of the alveolar cell membrane. They also noted that myelin figures in the alveolar space were often closely associated with the alveolar cell surface. All these observations supported an exocrine function for the great alveolar cell.

Studies of fetal lung development led many investigators to speculate that the inclusion bodies were the source

of surfactant. Buckingham and Avery (1962) found that normal surface activity of fetal mouse lung extracts appeared one day before term (18 days gestation). At 17.6 days gestation, Pattle (1961) noted that bubbles expressed from mouse lung were stable. Electron microscopic studies by Woodside and Dalton (1958) indicated that inclusion bodies first appeared in great alveolar cells of mouse lung at 18 days gestation. Balis and Conen (1964) found a maximum number of inclusion bodies in great alveolar cells of rats immediately before birth. Buckingham et al. (1964) showed that normal surface activity of lining films extracted from fetal rats developed two days before birth. Electron micrographs prepared from lungs of littermates demonstrated that the first appearance of great alveolar cell osmiophilic inclusions also developed two days before birth. Kikkawa et al. (1965) and Orzalesi et al. (1965) related the appearance of surfactant in the lamb to the presence of osmiophilic inclusions.

Kikkawa et al. (1965) and Weibel (1967) found that the periodic spacings of inclusion body lamellae coincided with the periodicity of laminations in phospholipid water complexes demonstrated by Stoeckenuis (1962). In a later study Kikkawa et al. (1968), compared lungs of fetal and newborn rabbits. They found that the inclusion bodies became markedly less osmiophilic after the onset of breathing. Inclusions in lungs of adult rabbits also showed a relative decrease in osmiophilia. "This alteration of inclusions

between fetal and newborn lung had not been recognized previously. With the support of biochemical and surface-physical results, this alteration was interpreted as indicative of the massive discharge of the inclusions of the Type II epithelial cells following the onset of breathing."

Using adult rabbits, Buckingham et al. (1966) showed by autoradiography that the cytoplasm of great alveolar cells represented the most active site of phospholipid synthesis in the lung. The labeled precursors, palmitate and acetate rapidly accumulated between larger inclusion bodies of the cell cytoplasm.

Kikkawa et al. (1958) and Sorokin (1967) tentatively concluded that synthesis of surface-active lipids occurred within the inclusion bodies. Sorokin (1967) presented a very interesting proposal for the origin and function of the lamellar inclusions or "cytosomes," which in the terminology of electron microscopists designated dense and polymorphic bodies believed to be lysosomes (Straus, 1967). Sorokin (1967) suggested that protein, which included precursors of enzyme systems necessary for lipid synthesis, was produced on the ribosomes attached to the cisternal membranes of the endoplasmic reticulum. These proteins were then released into the cisternal membrane subsequently incorporated by the Golgi apparatus. Next, these materials were pinched off to form vesicles [surrounded by a limiting membrane of monolayer dimensions (Suzuki, 1966b)]. The vesicles fused to form

multivesicular bodies [interpreted by Gordon et al. (1965) as an incorporation of small vesicles which are enzyme carriers derived from the Golgi apparatus] which ultimately joined together or became incorporated into cytosomes. He postulated that the lamellar appearance of inclusions represented "agranular membranes" pinched off from the Golgi apparatus to form vesicles. The membranes of the vesicles then "became incorporated into multivesicular bodies" and were responsible for their and the ultimate cytosomes' lamellar characteristics. Sorokin (1967) concluded that synthesis of cytosomal lipid occurred on these membranes.

Evidence against the proposal that great alveolar cell lipid synthesis occurred in cytosomes derived from transformed mitochondria (Klaus et al., 1962) was presented by Kikkawa et al. (1968). In their studies on fetal lung of rabbits, they observed an increased number of inclusion bodies with increasing fetal maturation. However, the number of mitochondria remained stationary. They also did not observe any inclusions with cristae similar to those found in mitochondria. Further, none of the mitochondria showed osmiophilia.

Suzuki (1966b), Kikkawa et al. (1968), Kuhn (1968), and Sorokin (1967), did not find transitions between mitochondria and lamellar inclusions. They did, however, observe cytoplasmic structures intermediate between multivesicular bodies and the multilaminar cytosomes.

The information presented above established a camp of thought which supported the great alveolar cell as the site of surfactant synthesis and secretion.

Niden (1967) presented evidence that shook the foundations of the great alveolar cell camp. He proposed that the nonciliated bronchiolar cells (Clara cells) lining the terminal airways actively secrete surfactant. The function of the great alveolar cell, he believed, was to engage in active phagocytosis of lipids and other materials existing in the alveolar spaces. Niden based this proposal on his observations of mice exposed to aerosolized carbon. Sequential electron microscopic analysis of lung tissue showed: (1) great alveolar cells actively phagocytized carbon particles and lipid materials from the alveolar space; (2) lamellar bodies originated from ingested lipids and were not secretory products; (3) the presence of the hydrolytic enzyme acid phosphatase at the lamellar inclusion body membranes indicated that these cytosomes of great alveolar cells were "lysosomes, that is phagocytic vacuoles containing acid hydrolysates"; (4) the Clara cells showed no evidence of phagocytic activity but, were active secretory cells.

Secretion granules of the Clara cell appeared to form in the Golgi apparatus and were subsequently extruded from cell's apex. These secretory droplets did not stain with periodic acid-Schiff, Alcian blue, and Sudan black. This suggested the absence of mucopolysaccharides, acid mucopolysaccharides, and free lipids. However, positive staining

with Sudan black-acetone, Baker's acid hematin, and aqueous silver hydroxylamine indicated that phospholipid was present.

Autoradiographic analysis five minutes after injection of labeled palmitate and acetate revealed positive silver grains at Clara cell apices. Niden suggested that great alveolar cell radioactivity was due to ingestion of phospholipid previously secreted by Clara cells.

Sorokin (1968) in discussing a paper presented by Niden (1968a) indicated that lysosomes can have a dual function: (1) phagocytosis and (2) expulsion of "unwanted" materials. Thus, the great alveolar cell lysosomal mechanism can be engaged in phagocytosis and also develop secretory bodies to be extruded. Niden (1968b) in response to these comments, suggested that materials secreted by Clara cells may possibly be taken up by great alveolar cells, transformed and then resecreted as surfactant. Kikkawa et al. (1968) indicated that the acid phosphatase activity in lysosomal mechanisms of great alveolar cells may be the result of secretory and not hydrolytic activity--or, a combination of both.

Goldsmith (1968) suggested that surfactant synthesis and secretion did not necessarily have to be an "either-or" situation. He proposed that both the Clara cell and the great alveolar cell may possibly function in this manner. To ease the current controversy, he suggested that further research should explore this possibility.

CYTOCHEMICAL EVIDENCE FOR OXIDATIVE AND SYNTHETIC PATHWAYS IN THE GREAT ALVEOLAR CELL

Identification of great alveolar cells in routine paraffin or frozen sections has proved to be a difficult task. As a result, few histochemical studies regarding their cytochemical characteristics have been reported. They are, however, somewhat conspicuous after staining frozen sections with Sudan black B, after incubation of fresh-frozen sections for nicotinamide-adenine dinucleotide phosphate (NADP or TPN) diaphorase, or after using the PAS method on 1 to 2 μ thick Epon-embedded sections (Sorokin, 1967).

Histochemical methods, although mainly qualitative at this time, permit precise localization of chemical activities among an organ's component tissues and cells (Sorokin et al., 1959, Tyler and Pearse, 1965).

Much of the preceding material presented in this review supplied ample evidence that the lung is not merely a passive organ involved in gaseous exchange. Its function in lipid synthesis is pretty well established.

As mentioned above, the work of Buckingham et al. (1966), Kikkawa et al. (1968), and Sorokin (1967) indicated that great alveolar cell cytoplasm represented the active site of phospholipid synthesis in the lung. The role of certain phospholipids in pulmonary surface phenomena has also been reviewed.

Tyler and Pearse (1965) and Tyler *et al*. (1965) developed a technique which prepared fresh-frozen sections of distended lung without significant alterations in oxidative enzyme histochemical reactivity. This method also provided for better visualization of great alveolar cells. Fresh lung was tracheally injected *in situ*, or excised segments were injected transpleurally with a dilute gelatine solution (4%), congealed with cold, running water, and cut into small blocks. These segments were then quenched in liquid oxygen or nitrogen and cryostat sectioned for subsequent histochemical studies.

Their results indicated that the great alveolar cell had great potential for metabolic activity. They noted that although exact correlations between enzymes demonstrated by histochemical and biochemical methods were not established for all the enzyme systems in their study, speculation of operating metabolic pathways was feasible from their observations. It was suggested that active synthetic and energy producing pathways were fully functional in the great alveolar cell. These metabolic routes included the glycolytic scheme (represented by lactate and α-glycerophosphate dehydrogenases), tricarboxylic acid cycle (represented by malate, succinate, and isocitrate dehydrogenases), and the pentose cycle (represented by glucose-6-phosphate dehydrogenase). They also identified strong reactions for NADP diaphorase

and nicotinamide-adenine dinucleotide (NAD) diaphorase. These enzymes are involved with biosynthetic systems.

These cytochemical observations with histochemical methods were confirmed by Said et al. (1966), Azzopardi and Thurlbeck (1967), and Sorokin (1967).

Sorokin (1967) suggested that glyceraldehyde-3-phosphate dehydrogenase cytochemical activity was a specific reflection of a functional glycolytic pathway in the great alveolar cell. Reiss (1968) suggested that α-glycerophosphate dehydrogenase activity as revealed by histochemistry was not always indicative of glycolysis.

Said et al. (1966) found a reduced number in or diminished cytochemical reactivity of great alveolar cells after experimental pulmonary artery ligation of normal dogs. The possible association of the great alveolar cell biosynthetic pathways and surfactant production was suggested.

RENEWAL OF GREAT ALVEOLAR CELLS

Bertalanffy and Leblond (1953) stated that the mitotic activity of the lung indicated "a continuous renewal of the two types of alveolar cells." Under normal conditions, loss by desquamation into the air passages and proliferation by mitosis occurred in a steady state. The lifetime of great alveolar cells was calculated to be approximately 7-8 days (Bertalanffy, 1967).

Evidence has been presented by Bils and Romanovsky (1967) and Sherwin et al. (1967) that conditions of stress, e.g., oxygen toxicity, air pollution and animal ageing may alter the dynamic equilibrium which maintains a relatively constant number of great alveolar cells.

The question of great alveolar cell renewal as the basis for surfactant turnover was discussed by Clements (1967a). He concluded that the relatively slow cell turnover compared to surfactant's high rate of flux probably excluded alveolar cell renewal as the control mechanism for adequate surfactant production. He added, however, that further investigation of such control mechanisms may "well prove to be a fascinating chapter in pulmonary physiology."

A careful review of the literature did not reveal any studies (other than pulmonary artery ligation, air pollutants, or ageing effects) relating lung populations of either the great alveolar or the Clara cells to acute or chronic physiologic stress.

THE EPHEMERAL NATURE OF THE SURFACE LINING

Williams et al. (1966) found that in experimental animals pulmonary compliance decreased gradually with prolonged shallow breathing. They concluded that the alveoli could not maintain low surface tensions for long periods without intermittent large inflations. The deep ventilations, they suggested replenished the air-alveolar

interface with surface-active materials. Scarpelli (1968) pointed out that during alveolar lining compression surfactant leaves the surface ("insoluble" phase) and enters the bulk or hypophase. Surfactant is returned to the surface on expansion. He suggests that the maintenance of normal surface activity depends upon normal inspiratory and expiratory cycles.

Faridy et al. (1966) and Permutt (1966) found that ventilation of excised lung produced a decrease in percent lung volume at a given transpulmonary pressure. The reduced compliance was directly related to the tidal volume and duration of ventilation. There was an inverse relationship to the end-expiratory pressure.

Clements (1967a) and Scarpelli (1968) estimated that the surfactant turnover rate was about fifteen to eighteen hours. When compared to metabolic activities of other substances in the body (e.g., myelin lipids of brain have a half-life of about one year), Clements (1967a) stated that the alveolar lining was "truly evanescent."

MATERIALS AND METHODS

Initially, 72 male, albino Sprague-Dawly rats 100 days old were housed in sedentary cages for a seven-day environmental adjustment period. Sixty of these animals were later selected on the basis of weight uniformity. They were then matched in trios by weight and randomly assigned to one of three treatment groups.

ANIMAL GROUPS

1. A sedentary group comprised of rats housed in sedentary cages (24 x 18 x 18 cm) for the duration of the experiment. The rats were removed from their cages once a week for body weight determinations.

2. A sedentary-forced group was also housed in sedentary cages. However, these animals had one thirty-minute swim per day with a weight equal to three per cent of their body weight attached to the tips of their tails with miniature plastic clothespins. Placement of weights in this position, rather than at the tail base, reduced the buoyance effect of air trapped in the fur. This effective reduction forced the rats to exercise more strenuously during the swimming periods.

3. A voluntary-forced group was housed in cages equipped with voluntary exercise wheels. In addition to their free choice of wheel exercise, this group was forced to swim for two thirty-minute periods per day with weights equaling three per cent of their body weight attached to the tail tips.

PROCEDURES

Rats were swum in individual cylindrical tanks measuring eleven inches in diameter and thirty inches deep. Following each swim, the animals were towel-dried and returned to their cages.

To facilitate later sacrifice procedures, two different trios were selected each day for ten consecutive days during the initial phases of the program. This allowed for a similar order of sacrifice during ten consecutive days when the experimental treatments were terminated. In this way, the duration of all animal treatments was held constant.

The sedentary-forced group had one 30 min. swimming period per day between the hours of 9:00 a.m. and 12:00 noon. The animals were exposed to this activity six days per week for 52 days. The voluntary-forced group had two separate 30 min. swimming sessions per day, six days a week for 52 days between 9:00 a.m. and 12:00 noon, and again between 5:00 p.m. and 8:00 p.m. In both groups, the lead weights were adjusted weekly to changing body weights.

To allow for the animals' adaptation to the swimming regimen, no weights were attached during the first two days of forced exercise.

An animal submerged for 10 seconds below the surface of the water was used as a criterion for fatigue (McArdle and Montoye, 1966). When this occurred, the fatigued animal was rested briefly, and then subjected to the forced exercise once again. Four rats died during the experimental period. Three of these were sedentary-forced, and one was a voluntary-forced animal. Five rats showed both gross and histologic evidence of pulmonary pathology (one sedentary, two sedentary-forced, and two voluntary-forced). These animals were not included in subsequent data retrieval.

Each animal was fed ad libitum from a stock diet. Conditions of room temperature, humidity, and air exchange were maintained at recommended levels. The temperature of the water used in the swimming tanks ranged from 32° to $34^{\circ}C$.

The day following each final treatment, the animals were anesthetized intraperitoneally with pentobarbital sodium (Halatal). The thoracic cavity was opened and a ligature placed between the superior and middle lobes of the right lung. The superior lobe was carefully excised and used for bubble stability measurements which will be described later.

The trachea was isolated in the cervical region, cannulated and four per cent gelatin in Ringer's solution at

37°C. with pH adjusted to 7.6 as suggested by Azzopardi and Thurlbeck (1967) was injected until the volume of the lungs filled the thoracic cavity. The gelatin in the lungs was hardened by cooling in running cold water for approximately five minutes (Tyler and Pearse, 1965). Small segments of gelatin-infiltrated inferior lobes of the right lung were placed on chucks and frozen in isopentane which had previously been cooled to a viscous fluid with liquid nitrogen. These small blocks were placed in an Ames Lab.-Tek microtome cryostat maintained at -15°C., and within three hours cut at 8μ for subsequent histochemical staining.

Estimates of pulmonary surfactant were made using the Pattle (1958) bubble method. Small segments of the superior right lobe were inflated by transpleural air injections using a syringe equipped with a 27-gauge needle. The inflated lung tissue was squeezed with a Crile forceps and the extruded bubbles transferred into a drop of distilled water hanging under a microscope slide. This slide was then placed over a glass concavity slide designed for hanging drop preparations. The stability ratio (Sr), defined as the ratio of the final surface area to the initial surface area, was determined by dividing the sum of the squares of the diameters of a group of at least 10 bubbles after twenty minutes by the sum of the squares of the initial diameters of those bubbles. According to Pattle (1966), the Sr is a measure of the amount of surface-active lining film the

bubbles have brought from the alveoli. Bubbles with initial diameters ranging from 35 μ to 60 μ were measured with an ocular disc micrometer. The micrometer was calibrated by use of an A0 Spencer Bright-Line Hemacytometer. One division on the disc micrometer scale was equal to 10.2 μ. A field of selected bubbles was rapidly sketched. Immediately thereafter, the microscope light was turned off. Periodically the light was turned on briefly to keep track of bubble orientation for final measurements. Mean stability ratios were calculated for each animal and these values were then compared among the animal groups using a one-way analysis of variance (Table 1).

The work of Bertalanffy and Leblond (1953) suggested that the normal appearance and number of alveolar cells in alveolar tissue was the result of a dynamic equilibrium between their proliferation by mitosis and their loss by desquamation into the air passages. More recently, Sherwin et al. (1967), using lactate dehydrogenase histochemical reactivity as a marker for alveolar cells, determined the ratio of alveolar cells to alveoli after lung exposure to low levels of air pollutants. In our laboratory, this technique was modified by using NADP diaphorase reactivity (sometimes referred to as TPND), as a marker for the alveolar cells (Fig. 1). Mean alveolar cell-alveolar ratios were determined by counting at least two different fields per slide. These cell-alveolar counts were made by use of a ground glass

screen five and one-half inches square attached to an ortho-lux microscope at a total magnification of 380x. Stage micrometer readings were recorded for each field enabling reliability measurements to be made. The mean ratios were compared among the three animal groups using a one-way analysis of variance (Table 2). NADP diaphorase was selected as a marker because of its intense cytochemical reactivity in great alveolar cells as previously reported by Tyler and Pearse (1965) and Sorokin (1968). It has been suggested that this reactivity reflects an active hexose monophosphate shunt generating reduced NADP necessary for the synthesis of licithin (Said et al., 1966) which is believed to be the essential lung surfactant (Clutario et al., 1966).

A final procedure was employed using histochemical methods described by Pearse (1961), Barka and Anderson (1963), Tyler and Pearse (1965), and Azzopardi and Thurlbeck (1967). Tissue for the histochemical procedures was obtained as described in a preceding section of this paper.

Histochemical reactivity which may reflect cellular enzyme activity was qualitatively evaluated in great alveolar cells with the light microscope. The oxidative enzymes histochemically demonstrated were: glucose-6-phosphate dehydrogenase (pentose cycle), α-glycerophosphate and lactic acid dehydrogenases (glycolytic pathway), succinic and malic acid dehydrogenases (tricarboxylic acid cycle), nicotinamide-adenine dinucleotide phosphate diaphorase, and nicotinamide-adenine dinucleotide diaphorase.

The biological oxidation and reduction mechanisms involving hydrogen and electron transfer through one or more intermediates to oxygen provide a major energy source in the living cell (White et al., 1964).

These mechanisms are controlled by the enzymes of biological oxidation. Dehydrogenases are oxidative enzymes which activate specific hydrogen atoms of a substrate and catalyze their transfer to a hydrogen acceptor or to a consecutive series of acceptors. Other than succinate dehydrogenase, each dehydrogenase is either NAD or NADP dependent. When a dehydrogenase acts upon a given substrate, the substrate is oxidized (loses electrons) and hydrogen is released. The hydrogen atom is then taken up by the coenzymes NAD or NADP. In the process, the coenzymes are reduced (gain electrons) to NADH or NADPH and subsequently reoxidized by their respective and specific flavoprotein diaphorases (NAD-diaphorase and NADP-diaphorase). The flavoproteins are then capable of passing the activated hydrogen into the electron transport chain.

Succinate dehydrogenase is the only histochemically demonstrable enzyme which requires no coenzyme to transfer an activated hydrogen to the electron transport chain (Pearse, 1961).

The histochemical methods used for these enzymes depend upon the formation of a dense, colored precipitate at the supposed site of enzymic activity. Precipitate formation

results from the reduction of a water-soluble tetrazolium salt (quaternary ammonium salt) to a water-insoluble, colored formazan. Thus, in the histochemical reaction, the tetrazolium salt acts as an electron acceptor (Barka and Anderson, 1963). The electrons, if not intercepted, would usually pass by way of a succession of carriers to molecular oxygen. The efficacy of tetrazolium salts depends upon their ease of reduction (redox potential, E^{o}, or oxidizing capacity), solubility, molecular size, charge, and light sensitivity (Pearse, 1961).

In all the histochemical methods for dehydrogenases, the tetrazolium salt, 2,2′-di-p-nitrophenyl-5,5′-diphenyl-3,3′-(3,3′-dimethoxy-4,4′-biphenylene) ditetrazolium chloride (Nitro blue tetrazolium, NBT, or Nitro-BT) was used.

Methods for NAD and NADP linked dehydrogenases demonstrated in this study were modified from Barka and Anderson (1963), Hess et al. (1958), Nachlas et al. (1958a and b), Tyler and Pearse (1965), and Azzopardi and Thurlbeck (1967).

The incubation media contained:

Appropriate stock solution, 1.0 M	1 ml
NAD or NADP (4 to 6 mg/ml)	1 ml
Respiratory inhibitor (sodium azide, 0.1 M)	1 ml
Magnesium chloride, 0.05 M	1 ml
Buffer, 0.2 M phosphate (for lactate dehydrogenase), 0.2 M tris (for all others); buffer pH 7.4-7.6	2.5 ml
Nitro-BT (4 mg/ml)	1 ml
Distilled water	1 ml
Menadione (as a hydrogen carrier for α-glycerophosphate dehydrogenase), 4 mg/ml	1 ml

The various substrates and corresponding coenzymes used were:

Dehydrogenase	Substrate	Coenzyme
Malate	Sodium L-malate	NAD
α-glycerophosphate	Sodium DL-α-glycerophosphate	NAD
Lactate	Sodium DL-lactate	NAD
Glucose-6-phosphate	Glucose-6-phosphate, disodium	NADP

Incubation time for sections was 30 min. at 37°C.

The incubation medium for succinate dehydrogenase was modified from Barka and Anderson (1963) and Azzopardi and Thurlbeck (1967) as follows:

Sodium succinate (substrate), 0.06 M	1 ml
Nitro-BT, 0.2%	2.5 ml
Menadione (as a hydrogen carrier), 4 mg/ml	1 ml
Phosphate buffer, 0.2 M (pH 7.4)	1 ml
Ringer solution	0.5 ml

Sections were incubated for 30 min. at 37°C.

Incubation media were freshly prepared each day. The medium and conditions of time and temperature were kept constant for each specific enzyme demonstrated. Subjective evaluations of formazan color intensity were made only on sections retrieved from animals sacrificed on a given day. Incubation media lacking substrate or coenzyme were periodically used as controls for all enzymes studied. Heart, muscle, and nerve tissue were incubated with lung sections under exactly the same conditions to serve as positive controls for many of the enzymatic tests.

Hematoxylin and eosin stained sections were prepared from all the specimens studied for routine demonstration of tissue components.

The relative intensity of the reaction in great alveolar cells was estimated at 400X and recorded on an arbitrary scale expressed as ++++ for very strong. Weaker reactions were given successively lower gradings down to + for very weak intensity and 0 for absence of reaction.

After a number of evaluations, it was apparent that the histochemical reactions in great alveolar cells found in serial sections from the same animal were uniform in intensity. As a result, the individual cell reaction intensities were estimated and a mean intensity for all cells in each section was recorded (Appendix A).

Statistical analyses of the subjective evaluations were not carried out because of the very evident inherent variables existing in histochemical techniques of this nature. Visual estimations of color intensity are at best semi-quantitative. Compounded to this are the variables resulting from: differences in the thickness of parallel cryostat sections; close-packing of formazan granules in the cytoplasm of one cell and dispersion of approximately the same number of granules in a second cell; a different visual field for intensity estimation; possible diffusion of granules resulting in false localization (Glick, 1961); staining "competition" from similar enzyme systems at different locations in the same cell and in other cell types existing in the same tissue. These are but a few of the variables which preclude quantitative interpretations and the

successful application of statistical methods when gradations of light and dark reaction intensities are visually estimated (Azzopardi, 1968, Niden, 1968c, Pearse, 1968, Tyler, 1968).

RELIABILITY DATA

The cells:alveoli ratios were later redetermined for six animals randomly selected from each of the three treatment groups.

The per cent correlation between the initial and subsequent ratios was determined (Appendix C).

Similar linear correlations were not possible for the bubble stability ratios nor practical for the subjective histochemical evaluations.

RESULTS AND DISCUSSION

BUBBLE STABILITY RATIOS (Sr) IN THE THREE ANIMAL GROUPS

The mean stability ratio for each of the treatment groups is indicated in table 1. Mean initial and final bubble diameters for each animal in the three groups are given in Appendix B.

No significant differences at the 0.05 level were evident among the groups. There was, however, a trend toward progressively lower $\overline{Sr}$ values with increased levels of physical activity.

In excised lungs, Faridy et al. (1966) found that when the lobes were ventilated, there was a decrease in the percent volume at a given transpulmonary pressure. They noted that the decrease was directly related to the tidal volume and duration of ventilation. It was also apparent that the ventilation effects were reversed if the lobes were kept at a constant volume for several hours at optimum temperatures and appropriate levels of oxygen tension. They suggested that recovery was due to an active process. It was postulated that ventilation decreased the amount of surfactant at the air-alveolar interface. However, if the cells responsible for surfactant synthesis and secretion were

adequately supplied with essential precursors and functioning normally, more surfactant would be produced and replace that diminished by ventilation.

Permutt (1966) concluded that if "surfactant is depleted by ventilation, one must conclude that during life under normal conditions a balance is maintained between depletion and regeneration. The oxygen tension and temperature of the alveoli appear important in determining the rate of regeneration."

Clements (1967c) suggested that surfactant was not perfectly stable under both *in vivo* and *in vitro* conditions. He indicated that a spontaneous decrease in pulmonary compliance occurred in both healthy and diseased lungs. However, since surfactant normally had a half-life of about half a day, there was probably a rapid response to excessive *in vivo* demands for replacement.

Scarpelli (1968) observed that the lung synthesizes phospholipids and presents them to the lining layer "extremely rapidly."

The $\overline{Sr}$ values obtained in this study support, by indirect evidence, the above findings. The demands placed on intact rat lung by the increased levels of chronic physical activity in this study were apparently not excessive enough to significantly alter bubble stability beyond gradations of "normal."

Table 1

Comparison of Mean Bubble Stability Ratios *($\overline{SR}$) in the Three Animal Groups

Group	$\overline{Sr}$	F	.05 Level
Sedentary	0.70	1.74	**NS
Sedentary-forced	0.61		
Voluntary-forced	0.57		

*$\overline{Sr}$ determined from each group's mean final bubble diameters squared ($\overline{FD}^2$), divided by mean initial bubble diameters squared ($\overline{ID}^2$).

Sr values represent measurements of at least 10 bubbles per each animal.

**NS: not significant.

GREAT ALVEOLAR CELL:ALVEOLI RATIOS IN THE THREE ANIMAL GROUPS

Table 2 gives mean great alveolar cell:alveoli ratios for each treatment group. The number of alveoli and great alveolar cells counted in two different fields per slide for each animal are included in Appendix B.

One way analysis of variance indicated no significant differences among the ratios calculated for each group.

This finding is consistent with the experimental evidence for great alveolar cell turnover versus surfactant turnover in normal animals. The estimated renewal interval

of about 12 to 15 hrs. for the surface lining (Clements, 1967a) is much shorter than the approximate 7 to 8 day lifetime of the great alveolar cell (Bertalanffy and Leblond, 1953, Blenkinsopp, 1967, Bertalanffy, 1967). This study demonstrates that prolonged exercise by swimming does not significantly increase the number of great alveolar cells per air space in rat lung.

With the assumption that such physiologic stress makes demands upon the *in vivo* integrity of surfactant, it is, according to Clements (1967a)"probably reasonable, therefore to exclude replacement of alveolar cells [great alveolar cells] as the basis for the turnover of dipalmitoyl lecithin [believed to be the essential surfactant in mammalian lung]."

Table 2

Comparison of Mean Great Alveolar Cell-Alveoli Ratios ($\overline{C}/A$) in the Three Animal Groups

Group	$\overline{C}/A$	F	0.5 Level
Sedentary	1.78	2.85	*NS
Sedentary-forced	1.50		
Voluntary-forced	1.64		

$\overline{C}/A$ values represent observations of at least two fields per slide per animal.

*NS: not significant.

Table 3

Per Cent Correlation from Reliability Data Obtained for Great Alveolar Cell:Alveoli Ratios ($\overline{C}/A$)

Treatment Group	% Correlation
Sedentary	95%
Sedentary-forced	98%
Voluntary-forced	98%

This table represents the per cent correlation between two separate determinations of $\overline{C}/A$. The separate ratios were completed on approximately one-third of the animals in each treatment group (Appendix C).

Histochemical methods

Table 4 represents a summary of estimated enzyme activity ranging from + for slight activity to ++++ for very intense activity; 0 indicates an absence of visible histochemical reactivity. Numerical values indicate the per cent of animals in each treatment group falling into a subjective activity category for each enzyme.

Table 5 indicates the frequency distributions of estimated activity for the following enzymes: NADP-diaphorase, NAD-diaphorase, malate dehydrogenase (MDH), α-glycerophosphate dehydrogenase (α-GPDH), lactate dehydrogenase (LDH), glucose-6-phosphate dehydrogenase (G-6-PDH), and succinate dehydrogenase (SDH).

Table 4

Per cent of Animals at Each Level of Estimated Activity*

Enzyme**	Sedentary				Sedentary-Forced				Voluntary-Forced			
	+	++	+++	++++	+	++	+++	++++	+	++	+++	++++
NADP-D	0	24	47	29	0	9	91	0	0	7	47	47
NAD-D	0	17	61	22	0	0	100	0	0	33	47	20
G-6-PDH	44	25	19	13	55	9	27	9	55	18	18	9
α-GDPH	0	12	35	53	0	0	69	31	0	0	64	36
LDH	0	19	56	25	0	8	62	31	0	33	33	33
MDH	6	59	24	12	0	44	44	11	17	3	50	0
SDH	33	64	7	0	22	66	11	0	8	69	23	0

*+ represents weakest activity, ++++ indicates strongest activity. Zero represents no apparent activity.

**Key to enzyme abbreviations: NADP-D, nicotinamide-adenine dinucleotide phosphate diaphorase; NAD-D, nicotinamide-adenine dinucleotide diaphorase; G-6-PDH, glucose-6-phosphate dehydrogenase; α-GPDH, α-glycerphosphate dehydrogenase; LDH, lactate dehydrogenase; MDH, malate dehydrogenase; SDH, succinate dehydrogenase.

Table 5

Frequency Distribution of Estimated Enzyme Activity in the Treatment Groups

Enzyme	*Reactivity	Sedentary Group	Sedentary-Forced Group	Voluntary-Forced Group
NADP-diaphorase	+1	0	0	0
	+2	4	1	1
	+3	8	10	7
	+4	5	0	7
NAD-diaphorase	+1	0	0	0
	+2	3	0	5
	+3	11	11	7
	+4	4	0	3
MDH	+1	1	0	2
	+2	10	4	4
	+3	4	4	6
	+4	2	1	0
α-GPDH	+1	0	0	0
	+2	2	0	0
	+3	6	9	9
	+4	9	4	5
LDH	+1	0	0	0
	+2	3	1	5
	+3	9	8	5
	+4	4	4	5
G-6-PDH	+1	7	6	6
	+2	4	1	2
	+3	3	3	2
	+4	2	1	1
SDH	+1	4	2	1
	+2	9	6	9
	+3	1	1	3
	+4	0	0	0

*Increasing reactivity is expressed by the arbitrary scale, +1 to +4. Zero indicates no apparent reactivity.

No unambiguous patterns were readily determined for any of the enzymes demonstrated in the three animal groups. There were indications of increased activities for NADP-diaphorase and NAD-diaphorase in the sedentary-forced group, and for α-glycerophosphate dehydrogenase in both the sedentary-forced and voluntary-forced groups.

The intense reactions for the diaphorases were expected on the basis of their participation in the hydrogen transfer chain of specific dehydrogenases frequently associated with biosynthetic systems (Tyler and Pearse, 1965, Azzopardi and Thurlbeck, 1967). In particular, increased NADP-diaphorase activity may reflect an enhanced pentose cycle generating reduced NADP necessary for fatty acid synthesis and the subsequent production of lecithin (Said et al., 1966).

The menadione technique for α-glycerophosphate dehydrogenase indiscriminately demonstrates both the intra (oxidase)- and extra-mitochondrial species of this enzyme. The increased activity noted may reflect enhanced mitochondrial α-glycerophosphate oxidase activity. "The function of the active lung mitochondrial L-alpha glycerophosphate oxidase may be related to a rapid production by glycolysis of alpha-glycerophosphate, which is needed for lecithin synthesis" (Reiss, 1966).

Localization of formazan granules followed a very consistent pattern in all the oxidative enzyme studies.

Heavy concentrations of granules were uniformly distributed throughout the cytoplasm (Fig. 2). This may reflect extensive mitochondrial distribution in great alveolar cells and associated high concentrations of oxidative enzymes (Sorokin, 1967).

Said et al. (1966) suggested that the abundance of oxidative and synthetic metabolic enzymes in great alveolar cells supported their proposed function in surfactant production. A paucity of these enzymes when pulmonary surfactant is abnormal lends further support to this concept.

A consistently high reaction intensity in bronchiolar epithelium compared to that found in the parenchyma was evident in all three animal groups (Fig. 3).

SUMMARY AND CONCLUSIONS

Sixty adult male rats were subjected to prolonged sedentary, sedentary-forced, and voluntary-forced levels of physical activity. Observations were made regarding the effects of these activity levels on: lung bubble stability ratios; great alveolar cell to alveolar ratios; and on the histochemical reactivity of some enzymes believed important in great alveolar cell metabolism.

No statistically significant differences were obtained for bubble stability ratios, nor great alveolar cell to alveolar ratios determined for the three animal groups. Subjective evaluations of histochemical activity indicated increased reaction intensities with increased levels of physical activity for NADP-diaphorase, NAD-diaphorase, and α-glycerophosphate dehydrogenase. It was difficult to determine characteristic patterns of reactivity for any of the other enzymes studied.

Bubble Stability Ratios

If the film lining the bubbles expressed from apparently healthy lung was originally the alveolar surface lining (Pattle, 1958), and, as Slavkovic *et al*. (1968) suggested, the bubble stability method is sensitive enough to

differentiate between gradations of normal, then the $\overline{Sr}$ values obtained in this study reflect by indirect evidence the rapid formation of adequate surfactant to meet the demands of varying levels of pulmonary ventilation.

Great Alveolar Cell/ Alveoli Ratios

This study has provided evidence that significant increases in numbers of great alveolar cells per unit of lung space do not occur in rats chronically exercised by swimming and voluntary activity regimens. This finding supports the belief by Clements (1967a) that extremely efficient but, as yet, unknown control mechanisms maintain surfactant renewal in the face of excessive physiologic demands placed on the lung.

In light of the proposal that the non-ciliated bronchiolar cell (Clara cell) may be involved in the synthesis and elaboration of surfactant (Niden, 1967), it may be of interest to investigate, in future studies, Clara cell/ alveolar ratios in similarly treated animals. Comparisons of these values with those obtained in this study may provide more insight into the active processes of surfactant production. Comparative determinations of cell and nuclear size and numbers of osmiophilic inclusion bodies in both cell types may also shed more light on the problem of surfactant synthesis and secretion.

Histochemical Studies

It should be emphasized once again that histochemical methods employing visual estimations of enzyme reactivity in single cells of a heterogeneous tissue are mainly qualitative. Pearse (1968) stated that in a system such as this "even with say 10 separate observers the application of statistical methods is of doubtful value." He also indicated that "methods having flourescent end points will be developed which will permit quantitative microspectro-photofluorimetry of single cells containing the (fluorescent) product of enzyme activity."

In light of this and the previously mentioned variables, the data obtained from the histochemical methods were interpreted with caution.

Pronounced cytoplasmic localization of formazan granules was constant in all three animal groups.

Increased reactivity of NADP diaphorase, NAD diaphorase, and α-glycerophosphate dehydrogenase in rats subjected to sedentary-swim and voluntary-swim regimens may reflect enhanced metabolic pathways leading to surfactant production. More quantitative histochemical methods need to be developed for precise and reliable evaluations of pulmonary enzyme reactivity.

The data received from all methods used in this study reflect the ability of healthy lung to maintain

adequate surfactant and alveolar stability in the face of stressful exercise regimens.

Finally, in regard to this author's experiences with surface activity estimations, it is felt appropriate to recall the comments of Pattle (1966) who stated that the path of those who try to investigate the lung lining is beset by numerous sources of error, and that the investigator can best seek to avoid these by asking himself: what is really being measured, and what information is it indicating about the lung? Can this information be checked by another method?

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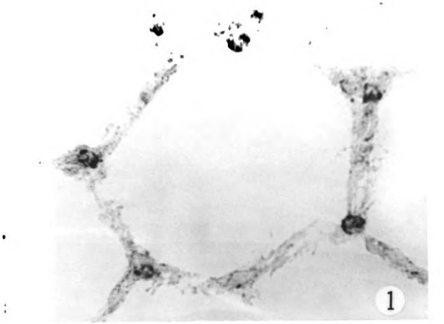
FIGURE 1

Fresh frozen section. Great alveolar cells distributed over alveolar walls show high reactivity for NADP diaphorase. Cells are either tucked away in a niche in the wall or bulge into the alveolar lumen. An alveolar macrophage is seen abutting against the wall. This represents a segment of a typical field used for cell-alveolar counts. Nitro blue tetrazolium, NADPH, pH 7.2, 30 min. at 37°C. (592 X).

FIGURE 2

Fresh frozen section showing a great alveolar cell with its lateral border exposed to the alveolar space. Formazan granules are heavily concentrated in the cytoplasm. The large nuclear area is relatively free of reactivity and shows vesicular characteristics. Faint reactivity is seen in other mural components. Nitro blue tetrazolium, NADPH, pH 7.2, 30 min. at 37°C. (1,480 X).

PLATE I



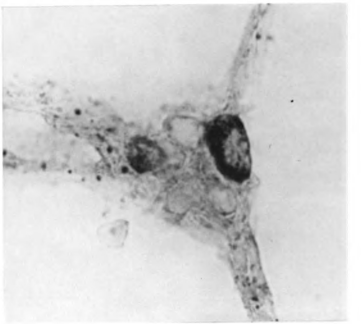
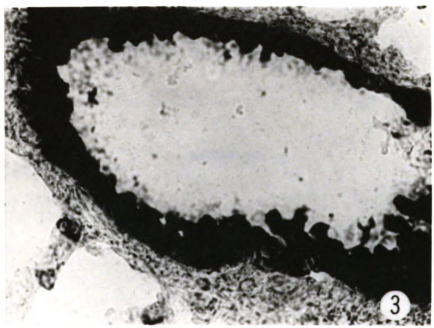


FIGURE 3

Fresh frozen section showing relative intensity of reaction in bronchiolar epithelium, parenchyma, and great alveolar cells. Nitro blue tetrazolium, NADH, pH 7.2, 30 min. at 37°C. (592 X).

PLATE II



APPENDIX A

BUBBLE STABILITY RATIOS ($\overline{Sr}$)

One way analysis of variance table

Source of variance	Df	SS	MSS	F
Among levels	2	0.1331	0.0665	1.74
Within levels	45	1.7194	0.0382	
Total	47	1.8525		

MEAN GREAT ALVEOLAR CELL/ALVEOLI RATIOS ($\overline{C}/A$)

One way analysis of variance table

Source of variance	Df	SS	MSS	F
Among levels	2	0.5279	0.2639	2.85
Within levels	36	3.3356	0.0926	
Total	38	3.8635		

Body Weight and Treatment Groups

One way analysis of variance

Source of variance	Df	SS	MSS	F
Among levels	2	80,975.12	40,487.56	40.30
Within levels	53	53,234.87	1,004.43	
Total	55	134,209.99		

APPENDIX B

*Mean initial ($\overline{ID}$) final ($\overline{FD}$) bubble diameters for each animal in the three treatment groups

Sedentary Group			Sedentary-Forced Group			Voluntary-Forced Group		
Animal Number	$\overline{ID}$	$\overline{FD}$	Animal Number	$\overline{ID}$	$\overline{FD}$	Animal Number	$\overline{ID}$	$\overline{FD}$
6	4.5	4.3	23	3.8	3.3	21	4.3	4.1
34	3.7	3.6	28	4.4	3.6	39	5.6	4.2
20	6.3	4.3	45	6.4	4.4	51	4.8	3.8
54	4.6	2.9	25	4.1	3.2	43	6.3	4.4
14	4.2	4.1	29	4.6	3.5	31	5.0	3.0
48	5.7	5.1	3	4.5	2.7	41	4.3	3.5
42	5.3	4.1	35	3.6	3.1	17	3.7	3.0
44	3.7	3.4	40	3.2	2.2	2	3.3	3.0
8	4.2	3.5	36	4.8	3.3	57	4.0	2.8
12	3.7	3.6	53	3.6	2.9	49	3.9	3.0
30	4.0	3.9	16	4.5	3.7	32	3.7	3.2
37	4.2	3.9	60	4.3	3.6	47	4.6	1.7
13	4.3	4.0	17	3.3	2.7	9	3.9	2.4
26	4.5	3.0	11	4.6	3.3	50	3.8	3.6
10	4.0	3.4	38	3.6	3.1	1	4.6	2.6
27	4.8	3.3				16	4.1	4.0
9	3.6	2.4						
52	3.6	3.0						
59	5.4	3.5						

*Mean values were calculated from at least 10 bubbles per animal.

GREAT ALVEOLAR CELL:ALVEOLI RATIOS ($\overline{C}$/A)
Sedentary Group

Animal Number	Microscope Field	Number of Alveoli	Number of Cells	$\overline{C}$/A
59	1	14	29	2.07
	2	15	28	
10	1	15	22	1.43
	2	15	21	
27	1	16	23	1.45
	2	15	22	
13	1	18	35	1.58
	2	17	28	
26	1	18	37	1.89
	2	19	33	
52	1	17	26	1.32
	2	13	18	
4	1	14	26	1.86
	2	14	26	
30	1	17	33	2.11
	2	15	34	
37	1	17	33	1.96
	2	14	30	
42	1	14	21	1.58
	2	12	20	

Animal Number	Microscope Field	Number of Alveoli	Number of Cells	$\overline{C}/A$
44	1	15	30	2.12
	2	10	23	
6	1	19	50	2.33
	2	23	48	
34	1	14	23	1.63
	2	16	26	
14	1	13	24	1.73
	2	17	28	
48	1	16	31	2.18
	2	11	28	
15	1	18	20	1.20
	2	15	17	
20	1	18	29	1.36
	2	18	20	

GREAT ALVEOLAR CELL:ALVEOLI RATIOS ($\overline{C}/A$)
Sedentary-Forced Group

Animal Number	Microscope Field	Number of Alveoli	Number of Cells	$\overline{C}/A$
38	1	14	22	1.58
	2	10	16	
53	1	17	28	1.46
	2	19	28	
11	1	20	28	1.21
	2	21	26	
24	1	20	39	1.85
	2	20	35	
40	1	20	29	1.38
	2	23	33	
55	1	13	22	1.71
	2	8	14	
45	1	11	24	2.00
	2	16	30	
29	1	15	24	1.51
	2	20	29	
46	1	14	21	1.35
	2	17	21	
60	1	16	18	1.12
	2	16	18	

Animal Number	Microscope Field	Number of Alveoli	Number of Cells	$\overline{C}/A$
36	1	17	26	1.42
	2	21	28	
22	1	16	26	1.53
	2	14	20	
28	1	17	22	1.30
	2	20	26	

GREAT ALVEOLAR CELL/ALVEOLI RATIOS ($\overline{C}$/A)
Voluntary-Forced Group

Animal Number	Microscope Field	Number of Alveoli	Number of Cells	$\overline{C}$/A
16	1	10	15	1.55
	2	10	16	
49	1	18	39	2.02
	2	18	34	
32	1	14	20	1.50
	2	12	19	
1	1	17	25	1.64
	2	16	29	
2	1	15	34	2.41
	2	19	48	
57	1	23	34	1.48
	2	22	33	
41	1	16	22	1.63
	2	19	35	
5	1	26	46	1.78
	2	20	36	
31	1	15	26	1.46
	2	26	34	
47	1	16	18	1.10
	2	15	16	

Animal Number	Microscope Field	Number of Alveoli	Number of Cells	$\overline{C}/A$
9	1	12	23	1.88
	2	12	22	
50	1	19	23	1.21
	2	19	23	
39	1	23	30	1.28
	2	17	21	

APPENDIX C

RELIABILITY DATA

Great Alveolar cell/alveoli ratios in two separate determinations on randomly selected animals from each treatment group

Sedentary Group		Sedentary-Forced Group		Voluntary-Forced Group	
C/A_1	C/A_2	C/A_1	C/A_2	C/A_1	C/A_2
1.45	1.45	1.57	1.57	2.17	2.11
2.10	1.90	1.25	1.25	1.43	1.38
1.53	1.53	1.95	1.95	1.47	1.35
1.94	1.94	1.45	1.40	1.48	1.45
1.94	1.94	1.69	1.78	1.77	1.69
1.50	1.43	1.60	1.60	1.95	1.95

BODY WEIGHTS (grams)

A		B		C	
Animal Number	Body Weight	Animal Number	Body Weight	Animal Number	Body Weight
6	400	7	Died	21	352
34	452	55	364	5	316
54	435	23	280	51	326
20	459	28	369	39	304
48	420	45	386	43	353
14	449	18	Died	58	Died
44	436	25	419	31	318
42	423	29	300	41	322
12	412	35	325	17	342
8	428	3	376	56	309
37	413	24	383	57	315
30	473	40	384	2	348
26	487	53	382	33	371
13	419	36	399	49	370
10	421	60	357	32	358
27	378	46	394	47	321
15	325	22	400	9	312
52	420	19	Died	50	348
59	438	38	394	16	329
4	398	11	404	1	325