Lawrence Berkeley National Laboratory
Recent Work

Title
THE PULMONARY ADENOMA OF THE MOUSE: A SIMPLE MODEL OF ITS GROWTH BASED ON SCANNING ELECTRON MICROSCOPY

Permalink
https://escholarship.org/uc/item/14k0375b

Author
Dole, Stuart R.

Publication Date
1977-05-01
THE PULMONARY ADENOMA OF THE MOUSE:
A SIMPLE MODEL OF ITS GROWTH BASED ON
SCANNING ELECTRON MICROSCOPY

Stuart R. Dole
Ph. D. thesis

May 1977

Prepared for the U. S. Energy Research and
Development Administration under Contract W-7405-ENG-48

For Reference

Not to be taken from this room
DISCLAIMER

This document was prepared as an account of work sponsored by the United States Government. While this document is believed to contain correct information, neither the United States Government nor any agency thereof, nor the Regents of the University of California, nor any of their employees, makes any warranty, express or implied, or assumes any legal responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by its trade name, trademark, manufacturer, or otherwise, does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof, or the Regents of the University of California. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof or the Regents of the University of California.
THE PULMONARY ADENOMA OF THE MOUSE:
A SIMPLE MODEL OF ITS GROWTH BASED ON
SCANNING ELECTRON MICROSCOPY

Stuart R. Dole
Ph. D. thesis

May 1977

Lawrence Berkeley California
University of California
Berkeley, California 94720

This work was done with support from the
U. S. Energy Research and Development Administration
THE PULMONARY ADENOMA OF THE MOUSE:
A SIMPLE MODEL OF ITS GROWTH BASED ON
SCANNING ELECTRON MICROSCOPY

Stuart R. Dole

ABSTRACT

This study of the urethane induced pulmonary adenoma of the alveolar
type 2 cell in strain A mice using the scanning electron microscope
showed that one of the presumably characteristic features of this tumor,
the compression of the surrounding tissue, to be an artifact of the
traditional fixation techniques. When the lung was fixed in the
inflated state rather than being allowed to collapse the tumors showed
no tendency to compress the surrounding tissue, but were of a clearly
locally invasive character.

This led to the idea that the tumor cells are motile. Exploring
some of the consequences of this a set of simple assumptions were
taken: (1) that the individual cells, in the absence of other
constraints, will move in the volume of the lung in a three-dimensional
random walk, (2) that the cells, in the absence of other constraints,
will multiply exponentially, and (3) that since the tumors, even in
their denser areas, show no signs of growth pressure, there is a cell
contact mediated inhibition of growth as the cells become more numerous.
The random walk is characterized by a root-mean-square step size per
unit time, and its probability density function is a three-dimensional
Gaussian distribution with a variance of the step size squared times
the number of steps. This is multiplied by the exponential growth function, characterized by the doubling time. The product then has the density limitation applied. The form of the density limitation is not critical.

This simple model correctly predicts the following characteristics of the growth of these tumors which have been described in the literature: (1) Average tumor radius is a linear function of time. (2) The presumably pre-tumor hyperplastic areas have an average radius that increases as the square root of time. (3) There is a time lag before the appearance of the tumors even though they appear to have been growing without appreciable latency. (4) The ratio of the largest to the smallest tumor radii remains fairly constant as the tumor population grows. The model also predicts the form of the tumor density as a function of radius seen in the scanning micrographs.

The conclusions that may be drawn from this study are that there are two mechanisms that can regulate the reproductive rate of these cells. One is some unknown process that keeps these cells in their normal sparse distribution. This is the mechanism the urethane exposure damages. The other is the cell contact mediated inhibition of growth that is normally dormant in these cells, but comes into play as the tumor becomes dense. Thus, this tumor is atypical of the solid tumors.

A series of stereo scanning micrographs of ethanol cryofractured tumors is included.
for Sarah
## CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>PREFACE</td>
<td></td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>BACKGROUND</td>
<td>4</td>
</tr>
<tr>
<td>The Lung</td>
<td>4</td>
</tr>
<tr>
<td>The Type 2 Cell</td>
<td>5</td>
</tr>
<tr>
<td>THE PROBLEM</td>
<td>8</td>
</tr>
<tr>
<td>The Alveologenic Adenoma of the Mouse</td>
<td>8</td>
</tr>
<tr>
<td>Urethane</td>
<td>10</td>
</tr>
<tr>
<td>Kinetics</td>
<td>12</td>
</tr>
<tr>
<td>Antigenicity</td>
<td>18</td>
</tr>
<tr>
<td>TECHNIQUE</td>
<td>19</td>
</tr>
<tr>
<td>Understanding</td>
<td>19</td>
</tr>
<tr>
<td>Scanning Electron Microscopy</td>
<td>22</td>
</tr>
<tr>
<td>Stereo</td>
<td>24</td>
</tr>
<tr>
<td>Transmission Electron Microscopy</td>
<td>25</td>
</tr>
<tr>
<td>Sample Preparation</td>
<td>26</td>
</tr>
<tr>
<td>RESULTS</td>
<td>29</td>
</tr>
<tr>
<td>Normal Tissue</td>
<td>29</td>
</tr>
<tr>
<td>Hyperplasia</td>
<td>30</td>
</tr>
<tr>
<td>Tumors</td>
<td>31</td>
</tr>
<tr>
<td>Panorama</td>
<td>36</td>
</tr>
<tr>
<td>Oddities</td>
<td>36</td>
</tr>
<tr>
<td>Perspective</td>
<td>38</td>
</tr>
<tr>
<td>Plates</td>
<td>39</td>
</tr>
<tr>
<td>Section</td>
<td>Page</td>
</tr>
<tr>
<td>---------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>90</td>
</tr>
<tr>
<td>Consequences of Motility</td>
<td>90</td>
</tr>
<tr>
<td>Random Motion</td>
<td>91</td>
</tr>
<tr>
<td>Growth</td>
<td>93</td>
</tr>
<tr>
<td>No Growth</td>
<td>94</td>
</tr>
<tr>
<td>Features of the Model</td>
<td>96</td>
</tr>
<tr>
<td>Linear Growth</td>
<td>99</td>
</tr>
<tr>
<td>Estimating S and T</td>
<td>100</td>
</tr>
<tr>
<td>SUMMING UP</td>
<td>107</td>
</tr>
<tr>
<td>The Model</td>
<td>107</td>
</tr>
<tr>
<td>What Happens</td>
<td>108</td>
</tr>
<tr>
<td>Remaining Problems</td>
<td>110</td>
</tr>
<tr>
<td>APPENDIX A: MATERIALS AND METHODS</td>
<td>112</td>
</tr>
<tr>
<td>APPENDIX B: REFERENCES</td>
<td>116</td>
</tr>
<tr>
<td>APPENDIX C: STEREO VIEWING</td>
<td>124</td>
</tr>
</tbody>
</table>
PREFACE

First of all, I would like to acknowledge those who have aided this work in its various phases. My committee members, Hardin Jones, Thomas Hayes, and Robert Glaeser, have perhaps been of the greatest material assistance. They have provided many fruitful hours of discussion and constructive criticism, and have shared their art, skill, and resources freely. I should like to especially thank Margaret White, for her help with the animals and her familiarity with the literature relating to the urethane induced adenoma, and James Pawley, for his assistance with the scanning microscopes and his stimulating intellect, and Francis Taylor, for her help with all phases of transmission electron microscopy. But most of all, I would like to offer my humble thanks to my spiritual teacher, Eknath Easwaran, and his teacher, Eknath Chippu Kunchi Ammal.

I would also like to sketch briefly the evolution of this project. It began as a scanning electron microscopic survey of the type 2 alveolar cell following a urethane exposure. I noticed that the tumors did not show the compression of surrounding tissue so often described in the literature. This led to the idea that the cells were motile. I spent several long weekends with a programmable pocket calculator simulating random motion in a lung-like matrix. I then found the excellent fit of some previously published data to the idea of random motion (illustrated in figure 2). With this inspiration I put the rest of the model together and verified the other predictions with the data. I have found the programmable calculator to be
invaluable for this sort of work. I was even able to implement a Regula Falsi algorithm to solve equation 8 for $S$ as a function of $T$. This work fell into place very rapidly after that.

This work was supported by the United States Energy Research and Development Administration contract W-7405-eng48 and the Environmental Protection Agency Award D5-E681 Subagreement 73 BAS.

Stuart R. Dole
Tomales, California
March 31, 1977
INTRODUCTION

In this work the urethane induced alveolar adenoma of the mouse is studied by scanning electron microscopy. By studying stereo electron micrographs of these tumors it becomes clear that some of the old ideas of how these adenomas grow are incorrect. Instead of growing as solid masses pushing the surrounding tissue away, they grow through the lung without disturbing its basic structure, by thickening the alveolar walls. This suggests that the tumor cells are infiltrative and motile. Exploring some of the implications of this motility, a simple mathematical model is devised that successfully describes many of the features of the growth and form of this type of tumor.

The alveolar adenoma of the mouse lung has been studied a great deal since its discovery near the end of the 19th century. It is common in several strains of mice, particularly the "A" strain. It is usually seen as a pearly white nodule, a few millimeters in diameter, just under the surface of the lung. It is formed by a single cell type, the alveolar type 2 cell, which is a cuboidal epithelial cell that secretes surfactant. It is normally a sparsely distributed solitary cell. In section, when prepared for light microscopy, the tumor shows an orderly adenomatous pattern, usually with a layer of compressed tissue around it. It is not encapsulated and grows slowly. The layer of compressed tissue turns out to be an artifact of preparation; the lung tissue is actually shrunken and stretched around the tumor since the lung is fixed in a collapsed state, and the surrounding tissue is not compressed at all. This is seen clearly in
the scanning electron micrographs. This artifact is easy to overlook since one would expect a growing tumor to push surrounding tissue out of the way and would not suspect that anything was amiss.

That the tumor grows without disturbing the lung implies that it is infiltrative. This is not a new idea since the tumor is known to be unencapsulated. This implies that the tumor cells must be able to move. While it is not feasible to observe it directly in the lung, mathematical exploration can give some clues to the nature of this motility. The simplest case is to assume that the motion is entirely random, like Brownian motion, since the mathematics of random motion are well established. Making the further assumption that the cell number will increase exponentially in the absence of inhibitory stimuli, and that there is some sort of density-mediated inhibition of growth when the cells become sufficiently crowded, a simple formula is derived that predicts most of the features of the growth and form of these tumors.

Most of the data used to develop the model comes from a study done in 1955 by Shimkin and Polissar. The model explains many of the features they describe. Briefly, their data show that (1) the average tumor radius is a linear function of time, (2) there is a lag from the time the carcinogen is administered until the tumors appear, even though the tumors seem to have been growing the whole time, (3) the radius of the presumably pre-tumor hyperplastic areas increases as the square root of time, and (4) there is a constant proportion between the radii of the larger and smaller tumors as they grow. Further, the model put forth in this thesis explains the density
distribution across the tumors seen in the scanning electron micrographs. Thus these simple assumptions turn out to be useful.

The alveologenic tumor of the mouse lung is atypical. The tumor seems to retain the cell-contact mediated inhibition of growth found in most normal solid tissues since it shows little evidence of growth pressure, but in the open structure of the lung it grows steadily. It grows by diffusion of its cells into the surrounding areas of the lung which act as seeds, multiplying and filling in the open spaces of the tissue and seeding further from there. Once an area has "filled in" it becomes a stable adenomatious tissue, with the original alveoli and airways becoming ducts and passageways, forming a glandular sort of arrangement. (The rate of division of the normal type 2 cells is not normally controlled by cell-contact inhibition, since they are solitary, but by some other mechanism. The event of transformation seems to be a release from this other mechanism.) None of the tumor becomes necrotic even at the center. It is a chemically induced tumor with the immunologic properties of spontaneous tumors and the growth characteristics of normal tissue. Its growth can be accurately described by a simple model with only two parameters, the step size characteristic of the random motion and the cell doubling time.
BACKGROUND

The Lung


The lung is the organ of exchange. Here the respiratory gasses, carbon dioxide and oxygen, are equilibrated between the blood and the atmosphere across the thin alveolar epithelium. For this the lung has a large surface area and a rich blood supply. The air spaces of the lungs communicate to the environment through the trachea and the branching airways. The airways terminate in myriads of small, nearly spherical cavities, the alveoli, which in the mouse are about 50 microns in diameter.

The entire air space is lined by a continuous epithelium. In the terminal bronchioles and alveoli this is composed of two cell types. The greatest area is covered by the type 1 cells, which are greatly extended squamous cells. The other cell type, which is actually a little more numerous, but comprising much less surface area, is the type 2 cell. This cuboidal secretory cell is also the stem cell for the type 1's. It is almost always found singly, tucked into the corners where two or three alveoli come together. It secretes the surfactant that reduces the surface tension of the air-water interface. Alveolar macrophages are also found in contact with the alveolar epithelium, and are sometimes found in close association with the tumors.
The Type 2 Cell

In the living state the alveoli are nearly spherical due to the large forces produced by the small radius of curvature of the air-water interface. This can be seen in Weibel's (1973) remarkable micrographs of vascular perfusion fixed lungs that were held at various degrees of inflation with air; even in nearly collapsed lungs the alveolar walls accordion and fold around the spherical air space. When spheres are packed together there are little spaces left at the junctures. It is in these spaces that the type 2 cell abides. It is cuboidal and fits there. In the samples of lung tissue examined in this study the air-water interface is destroyed by the process of fixation, so the alveoli assume a polyhedral shape, and the physical forces that constrain them in vivo are not apparent. Even though there are more type 2 cells than type 1's, they account for only a small fraction of the total surface area. Usually they are found singly except in cases of pathology (pairs are occasionally seen in normal tissue, however—see Bertalanffy 1964, Sorokin 1966).

The type 2 cell is metabolically highly active, its cytoplasm packed with organelles: well developed mitochondria, large numbers of multivescicular bodies, and the characteristic cytosomes (lamellar bodies) which are the sites of surfactant synthesis and storage. The structure and function of these cells is well described in the literature (see Sorokin 1970 & 1966, Smith & Ryan 1973, Ryan et al. 1975). Plates 6, 7, 8, 10, and 11 illustrate type 2 cells by transmission electron microscopy; these micrographs are from tumor tissue, but the
appearance of the cell changes little when it becomes cancerous (Brooks & Adkison 1971). Plates 2b through 2f illustrate a typical type 2 cell by scanning electron microscopy.

Typically, the cell has an apical surface exposed to an alveolus, covered with microvilli, but it may have two such surfaces on two different alveoli. The function of the microvilli is not known, but they may serve to scavenge excess surfactant material from the alveolus by analogy with the microvilli of the intestinal mucosa. The cell is usually anchored to the fiberous network of mesenchymal elements in the alveolar wall, and it has tight junctions (desmosomes) with the other epithelial cells it contacts. The cytoplasm has many well developed mitochondria, indicative of a great deal of metabolic activity. There is rough endoplasmic reticulum and some smooth, with a well developed Golgi apparatus. The unusually large number of multivesicular bodies found in the type 2 cell may arise from the smooth endoplasmic reticulum and Golgi apparatus. A complete spectrum of types can be found between the multivesicular bodies and the lamellar bodies, which are the final form of surfactant containing organelle prior to secretion. The lamellar bodies show the typical myelin form indicative of surfactant phospholipids. They can sometimes be seen discharging their contents into the alveolar lumen, and in scanning micrographs it is not uncommon to see small pores on the surface of the type 2 cells that may represent the same process.

The type 2 cell is known to have at least two major functions. The first is the synthesis, storage, and secretion of the surfactant needed to reduce the surface tension of the alveolar air-water
interface. The other is that it is the stem cell for the squamous type 1 cells (Adamson & Bowden 1974). In cases where the type 1 cells are injured, the type 2's multiply rapidly and in a few days the type 1's are replaced. The type 2's seem to be resistant to most of the noxious agents, notably oxidant gasses such as ozone or nitrogen dioxide, that destroy the type 1's (Bils 1974, Evans et al. 1973, Stephens et al. 1974). The response of the type 2's is swift yet limited. They are necessarily under a sensitive mechanism to control their reproductive rate. The type 2 cell also shows a proliferative response to most other forms of toxic lung injury (Witschi 1976).
THE PROBLEM

The Alveologenic Adenoma of the Mouse

One of the most common tumors in strain A mice is the adenoma of the alveolar type 2 cell. The extensive literature on this is well reviewed elsewhere (Shimkin 1955, Stewart et al. 1970, Shimkin & Stoner 1975). Some other mouse strains, such as the Swiss, share this, but others are almost entirely free from it. In the A strain, 40% will be bearing this tumor by the time they are a year old (Shimkin 1955). The tumors are relatively benign, rarely metastasize, grow slowly, and are probably not life limiting to the mouse. The tumors are locally invasive and are not encapsulated, which indicates a potential for malignancy. In other longer lived species the tumors are definitely malignant; in man the five year prognosis for survival is very poor, since the disease is usually well advanced before it is detected (Andrews & Morgan 1974).*

The tumor can be seen easily with the naked eye in the fixed collapsed mouse lung as a pearly white, spherical, well defined nodule that lies just under the pleural membrane. Often it lies near the surface of the lung, forming a raised lump. In section it has a well defined adenomatous pattern. In the usual preparation the surrounding tissue appears compressed by the tumor's growth. There is not an extensive degree of vascularization, and necrotic areas are not seen.

*This is a rare tumor in man, and is not to be confused with the much more common bronchogenic carcinoma associated with smoking.
In some cases there is further transformation; the tumor assumes a more typically sarcomatious appearance and exhibits metastasis. Virus particles are often seen in these secondary forms, but not in the primary adenomatious tumors (Kimura et al. 1972, Gross et al. 1976). The tumor may be serially transplanted, and it usually grows slowly. The typical picture is of a slowly growing, well differentiated, well structured tumor.

There is evidence in the recent literature (Menard et al. 1976, Rice 1969) that there may be two types of tumor involved, with most strains of mice only getting the relatively benign type described above, but the A and Swiss strains getting both. The other type differs in histologic pattern, grows faster, and shows more malignant characteristics. One instance of a clearly different sort of type 2 cell tumor was found in the scanning microscopy series (plates 17a and 17b).

The first description of this tumor was by Livingood in 1896, who came across it in an extensive series of autopsies of his mouse colony. It was believed to be bronchiolar rather than alveolar in origin, a belief that runs through the literature (see, for example Orr 1947 for bronchogenicity, Grady & Stewart 1940 for alveologenicity) until electron microscopy was able to clearly define the cell types involved (Klärner & Gieseking 1960, Svoboda 1962). Many instances of this spontaneous tumor were reported until around 1914, when interest waned (see review by Shimkin 1955). In 1925 pulmonary tumors were formed in mice by the application of coal tar distillates to the skin (Murphy & Sturm 1925). Later a whole galaxy of polycyclic aromatic
hydrocarbons were found to cause these tumors, along with nitrogen and sulphur mustards, some nitroso compounds and hydrazines, and alpha and gamma irradiation. But once again interest in this system waned until 1943 when it was found that urethane could also cause the alveologenic tumors.

Urethane

In the course of some investigations on the effects of X-rays on mice, Nettleship et al. (1943) used urethane as an anesthetic. The effects of the anesthetic alone were also measured, and a large crop of these tumors appeared. This was especially interesting since urethane is a very simple compound compared to the polycyclic hydrocarbons. It is safe to handle, water soluble, and relatively stable, characteristics not found with most carcinogens. Quantitative tumorogenesis and inferences based on dosimetry are easier with a simpler carcinogen such as this.

Urethane (ethyl carbamate, \(\text{NH}_2\text{-COOEt}\)) was first synthesized in 1834 by Duman. It has a large number of biological interactions (see Haddow 1963). It has long been used as an anesthetic, and is still used for this purpose in veterinary work. It is often used as a solvent for one of the barbiturates, but it is a useful anesthetic in its own right, as it produces satisfactory anesthesia without respiratory depression. Its mechanism of anesthesia is probably physical, like the other classical agents, such as xenon, nitrous oxide, halothane, etc., rather than neurochemical. It diffuses rapidly throughout the body's tissues, so the route of administration
None of the other anesthetics has the carcinogenic properties of urethane, even in the sensitive strain A mouse (Adenis et al. 1970), and urethane anesthesia antagonists, such as lysergic acid diethylamide, do not reduce lung tumor formation (see review by Haddow 1963). These two properties are unrelated; the anesthesia being due to physical properties of the molecule, and the carcinogenic properties being due to a chemical interaction with the cell's reproductive machinery (see Williams & Nery 1971 for a review of possible mechanisms). Urethane is a very potent carcinogen in this strain of mouse, though not in other strains, or species, where it is only mildly carcinogenic. It is capable of crossing the placental barrier and producing tumors in utero in the embryonic lung (Larsen 1947, also Nomura 1974a, b, c, Nomura & Okamoto 1972). Other tissues are also sensitive, such as thymus and liver (DiMarco et al. 1975, Bhide 1974, Haddow 1963), but they have a much slower response. Urethane seems to induce alveolar tumors without any detectable latency (see the regression line in figure 1).

Urethane is toxic to rapidly dividing cell populations in general, the thymus and immune system tissues being particularly sensitive (DiMarco et al. 1975). There is evidence that it interacts directly with DNA in some way, since labeled urethane can be shown to bind covalently to DNA (Bhide 1974, Ranadive 1973, Yarbro et al. 1967). Because it is toxic to rapidly dividing cells, it has been used as a chemotherapy agent against cancer (Haddow 1963), but it has been replaced with other agents with less severe side effects, which
include nausea, disorientation, and depression.

**Kinetics**

A number of investigations have been made into the kinetics of the induction and growth of the alveologenic tumor (Shimkin & Polissar 1955, Dyson & Heppleston 1975, 1976, White 1970, White et al. 1965, 1970, Kauffman 1972, 1974). The most interesting in regard to this study was Shimkin and Polissar's. They followed the number and sizes of tumors in a large number of mice following urethane exposure by careful examination of complete serial sections of one lobe. All tumors could be counted and measured, not just those visible on the surface. They also counted and measured "hyperplastic" areas, which were local areas of excess cellularity that were also associated with the urethane exposure. Most other studies have only dealt with tumors that were visible on the surface, or with the cellular kinetics of DNA synthesis or urethane metabolism for the lung as a whole. Most of the inspiration for this work comes from the purely physical aspects of tumor growth rate in Shimkin and Polissar's study.

The basic features that they found are the following. No tumors or clonal aggregations are seen for at least three weeks, when the first tumors, of some 70 microns diameter, can be seen by light microscopy in serial sections of a whole lobe. They contain about 400 cells. About this time the first hyperplastic areas are also seen. They are focal regions of excess cellularity. The number and size of the tumors increases thereafter; by about the seventh week the total number has reached a steady value, while the average size
increases steadily. The number and size of the hyperplastic areas increases at first, but then falls off. The number of tumors visible on the lung surface is always less than the number found by serial sectioning, and it levels off at about half that amount by 15 weeks. Most investigators have examined only surface tumors, which are a time-varying fraction of total tumors. While Shimkin and Polissar did not analyse their data this way, the average tumor radius as a function of time is fit very well by a linear function, and the average radius of the hyperplastic areas as a function of the square root of time gives a good linear fit up to the 12th week (see figures 1 and 2). The importance of this is discussed later.

One of the difficulties of this sort of study is that it is not possible to follow any one animal through time; to measure the tumors destroys the animal. Thus a statistical approach is necessary, taking many animals at each point to try to reduce the larger variance. Furthermore, since the tumors are appearing until about 50 days, the tumor population itself is changing during this time.

From Shimkin and Polissar's study it is found that there are about 400 cells in the earliest tumors, which appear at about three weeks. Since it is clear that these tumors are each derived from a single cell, and that the transformation probably takes place shortly after the urethane exposure, these cells must be dividing at least every 2.43 days \(2^{(21/2.43)} = 400\pm\). This is a generation time of about 58 hours. By using different techniques Dyson and Heppleston (1976) determined a cell cycle time of about 45 hours, but found that only a small fraction of the cells in the tumors were dividing 14 weeks
Figure 1. Tumor radius as a function of time, with data taken from Shimkin and Pollisar (1955). The o marks are the average radii derived from the geometric mean of the greatest cross sectional areas for each tumor, and the △ marks are the average of the three next-to-the-largest tumors, which represents a sort of maximum size. The two regression lines (A for the average tumors, and B for the largest tumors) are calculated to have the following values: for the average tumors, the y-intercept is .51 microns, and the slope 1.97 microns per day (coefficient of determination $r^2 = .99$), while for the largest tumors these values are -5.28, 2.77, and .95 respectively. The linear function fits the data very well. For the first two points the data are the same since there were only two tumors.
Figure 1.
Figure 2. Radii of the hyperplastic areas (Shimkin & Pollisar 1955) as a function of the square root of time. The bars are the extreme values found. The o's are the radii derived from the geometric mean of the cross sectional areas, and the ~'s are reported as "average". A is the regression line for the geometric averages from 21 to 105 days, and B the maximum sizes for 21 to 84 days.

The reason for plotting the data this way is that if the hyperplastic areas represent pre-tumor areas just becoming detectable, then the Gaussian distribution of the tumor cells predicted by the model will not be significantly perturbed by the density limiting factors and the size of the area will be proportional to the square root of time. The evidence for this is not very convincing, but suggestive enough to warrant further investigation. The largest areas give a good fit up to 84 days, and the average areas up to 105 days, but since the total number of tumors has become constant by about 50 days, the later points are of indeterminant significance.
Figure 2.
after urethane exposure. The normal alveolar wall cells were found to have a cell cycle time of about 30 hours. They also measured the growth of tumor size, but only measured surface tumors, which are a varying fraction of total tumors. The cell cycle times derived by the two methods agree reasonably well.

**Antigenicity**

The role of the immune system in cancer has been debated back and forth for many years (Prehn 1976). It has been suggested that since most spontaneous tumors are practically free from any detectable antigenicity, and since almost all model tumor systems using chemically or virally induced tumors in animals show considerable antigenicity, the greater portion of the current literature on tumor antigenicity may be of little value (Hewitt et al. 1976). In contrast to this, it appears that the urethane induced alveolar adenoma may be a special case, at least in the susceptible strains, in that it has very little antigenicity. It has recently been shown that in these strains normal tissue antigens occur that are only tumor associated antigens in other strains (Martin et al. 1973, 1976). This could explain the high incidence of these tumors in susceptible strains. There is, however, some evidence that these antigens are particularly associated with the faster growing "malignant" tumors (Menard et al. 1976). In any case, this should be cause for more interest in this tumor system, since it is so easy to work with, being chemically induced and of rapid response, yet having the immunological properties of the rare spontaneous tumors.
Understanding

The understanding of a problem depends largely on the techniques used to study it. In this case, the urethane induced alveolar adenoma of the mouse has been studied mostly by gross examination (tumor counting) and light microscopy. More recently transmission electron microscopy and the autoradiographic probes of cellular kinetics have also been used. The scanning electron microscope (SEM) has scarcely ever been applied (two SEM plates of a 1-carbonyl-2-phenylhydrazine induced alveolar tumor are presented in a paper by Toth & Shimizu 1974). While in careful morphometric work on lung tissue per se a great deal of attention is given to the degree of lung inflation at the time of tissue fixation (see Weibel 1973), in tumor work the lung is almost always fixed in the collapsed state. (For tumor counting the tumors stand out much better against a collapsed lung, and in sectioning there is much less "wasted" space on the slides.) On the other hand, for scanning work the open structure of the fully inflated lung is preferred. As it turns out, the usual techniques have introduced an artifact that has impeded understanding, especially since the artifact agrees with prior expectations. This is the shrinkage-compression artifact illustrated in Figure 3. As a further example, the direct imaging of the three dimensional relationships of the tumor with the surrounding structures has made clear some of the processes which were not obvious with other techniques. Viewing the scanning micrographs
Figure 3. The shrinkage compression artifact is schematically illustrated here. 3a shows the lung at a normal degree of inflation, with an embedded tumor a little below the pleural membrane. The tumor has grown by engulfing the lung structure without displacing it. The alveolar spaces are represented by the squares.

3b shows the same area after a 50% shrinkage in linear dimension. The lung shrinks elastically, but the tumor is solid, so the tissue becomes stretched around it, resembling compressed tissue. The tumor causes a raised area on the lung membrane.

Whenever the thoracic cavity is opened, or the lung removed from animal, it will collapse unless it is held inflated by positive pressure in the airways. In most preparations, the lung is fixed in this state. Thus upon histological examination the tissue around the tumors will appear to be compressed. As most tumors grow by displacing the surrounding tissue, this artifact would be overlooked since compressed tissue is expected around a tumor, and the invasive character of the tumor would not be so salient.
in stereo has been invaluable in this connection.

Scanning Electron Microscopy

The theory and use of the scanning electron microscope (SEM) are well covered in other works (Wells 1974, Hayes 1973). Only the features of its operation that are relevant to this work will be discussed. The SEM produces images that have a very natural appearance, like ordinary macroscopic objects in natural illumination. This quality of naturalness is further enhanced by using stereo views. The greatest advantage of the SEM, at least for this study, is the wealth of qualitative information it provides. For detailed quantitative work the transmission electron microscope (TEM) is preferred. It is this qualitative, natural approach that has provided the insights that have made this work possible.

The image produced on the console monitor cathode ray tube (CRT) of the SEM is not a true optical image, but is produced electronically. A focused beam of electrons is scanned across the sample's surface in a raster pattern, and the intensity of a synchronous beam scanned across the face of the CRT is modulated according to some measurable interaction of the primary beam with the sample. Typically, the primary beam has an energy in the range of 1 to 50 keV (10 keV is used in this study). When this strikes the specimen there are many sorts of interactions; some of the electrons are elastically scattered, others inelastically; large numbers of secondary electrons are produced, as are X-rays, and occasionally visible light. The usual imaging mode is to detect the secondary electrons and use their intensity to
modulate the brightness of the image. To be detected, a secondary electron must be produced close enough to the sample's surface to escape and reach the detector without being absorbed by another part of the sample along the way. Thus projections and edges will be "brightest", while hollows and cavities will be "darkest", as is the case with objects lit by ordinary light. Another less important contrast producing effect is the elemental composition of the sample since different elements have different secondary electron production coefficients, but in these samples the elemental composition is fairly constant, being a thin layer of gold-palladium alloy over the organic matrix. Also, electric charge may accumulate at various locations, due to an imbalance between absorbed and emitted electrons, which can deflect the trajectories of the secondaries, and even the primary beam. Efforts are made to reduce this effect by providing a conducting path to ground from all parts of the sample by means of the thin metallic coating.

The magnification of the SEM is the ratio of the area scanned by the electron beam on the CRT face to the area scanned on the sample. The magnification is limited by the spot size of the beam on the sample. In this study magnification in the range of about 50X to 15,000X were used, which compass the features of interest.

Most kinds of microscopy, especially those that use classical image forming techniques, view the specimens in thin section. A large amount of detail is visible this way, but it is often difficult to interpret since three dimensional information is entirely absent. The human visual mechanisms are adapted to perceive the surfaces of
whole objects, or the surfaces of cut objects where the relationships of the profiles of transected structures can be readily related to their actual forms. To understand the three dimensional structure of samples in thin section, many serial sections must be tediously obtained and carefully compared. Even so, misinterpretations are possible.

In the SEM, on the other hand, the images are much as people are used to seeing objects, with surface detail, enhanced edges, and shading of contours. The cognitive mechanisms man has developed to deal with the world of ordinary experience can be directly utilized.

**Stereo**

Stereo views are obtained by making two micrographs of the same area at slightly different angles, usually at a separation of 5 or 10 degrees to provide parallax, and then viewed such that each is seen by only one eye. When the images fuse, two things are observed to happen. One is of course that depth is perceived, and the other that there is some cancellation of the fine grain noise so that more features become apparent. There is evidently a correlative mechanism at work that extracts information by cancelling the uncorrelated noise it is buried in.

In this work, all the stereo views are made with a parallax of 5 degrees. The images are printed on the page so that the centers of the two views are about 65mm apart, which is the average interpupillary distance. To view them in stereo, the right eye must look at the right image, and the left at the left. This is often difficult since
the mechanisms in the eye for accommodation and convergence are coupled, but a pair of simple convex lenses can provide the necessary accommodation. The two lenses may be conveniently mounted in a simple holder (or one may be purchased) if very many pairs are to be viewed. The specifications of such a device are described in appendix C. See Boyde (1974a) for a discussion of stereo imaging with the SEM.

Transmission Electron Microscopy

Scanning electron microscopy is not a complete technique in itself and should be supplemented with traditional transmission electron microscopy to corroborate the interpretations. The TEM provides the only really positive means to identify cell type, and it is the only technique that clearly visualises the intracellular components. The theory and use of the transmission microscope are described elsewhere (Glauert 1974, Wischmitzer 1970). The methods used here are entirely standard.

The TEM has been extensively used to study this tumor, but most attention has been given to cellular and subcellular detail, rather than to the organization and structure of the tumor as a whole (see Klärner & Gieseking 1960, Svoboda 1962, Driessens et al. 1963, Kitamura 1964, Brooks 1968, 1970, Brooks & Adkison 1971, Kimura 1971). The general picture has been summarized in other sections, and the TEM plates taken for this study are typical of published work.
Sample Preparation

The techniques used to prepare the samples for the scanning electron microscope are specially devised to give the clearest interpretation of tissue structure (see Boyde 1974b). For viewing with the SEM, the sample must be completely dehydrated and free of volatile elements, since it is viewed in a vacuum, and it must be rendered somewhat conductive to reduce the charging artifact. Beyond this, means must be devised to preserve and display as much of the original structure as possible. The details of these processes are described in Appendix A. The general scheme is discussed here.

First of all, the tissue must be fixed (stabilized) so that it can be stored and processed without structural change. The lung tissue is fixed at inflation by introducing a gluteraldehyde–formaldehyde fixative into the airways at a pressure sufficient to produce full inflation without overdistention. This stabilizes the tissue by cross-linking the proteins. The reasons for fixing at inflation are to open the lung structure as much as possible and to try to approximate the in vivo structure. One artifact is introduced at this point, the destruction of the spherical shape of the alveoli produced by surface tension, but this is necessary if any detail is to be seen at all. The aqueous layer over the epithelium must be removed to reveal the underlying detail, and in the process the surface tension is lost and the alveoli become polyhedral. For transmission work this layer may be preserved by holding the lungs at inflation with air and fixing through the vasculature, but this is rarely done (see Weibel 1973). In most other tumor studies the lungs
have been fixed in the collapsed state, which distorts the tissue structure, especially around the tumors. See Hayat (1970) for a review of tissue fixation.

The fixed lungs are quite stable when stored in cacodylate buffer, and may be kept this way many months without detectable degradation. They are examined for tumors, which are excised along with control tissue. The tissue is trimmed so that the tumor is near the center of a small block, about the size of a long grain of rice. Then the tissue may be post-fixed with osmium tetroxide to stabilize the lipids and add color (the tumors turn black, while the rest of the lung is grey). This makes them visible for the fracture process which is carried out under conditions of poor visibility in liquid nitrogen.

The blocks are then dehydrated by passing them through a graded series of alcohol-water mixtures to 100% ethyl alcohol. Tissues for transmission work are taken off at this point and processed by the standard methods. The next step for the scanning samples is to fracture the blocks across the tumor to expose the inner structure while introducing as little damage as possible. A cut with a sharp razor causes much too much tearing. The Humphreys' technique (Humphreys et al. 1974) is used, where the sample, in alcohol, is frozen in liquid nitrogen and fractured as close to the tumor as possible. The Alcohol becomes a glass-like solid at this temperature, without producing crystals that could damage cellular structures. There is a good deal of uncertainty in this process, and only about half of the fractures actually pass through the tumor. The fracture plane passes smoothly through frozen alcohol and tissue alike, producing a
very clean cut with no torn edges. Often, both sides of the fracture are then available for examination, which aids interpretation. Aside from the uncertainty of where the fracture occurs, this method gives the best results of any that were tried.

The fractures blocks are transferred to acetone or amyl acetate from ethanol and dried by the critical point method (Anderson 1951, Boyde & Wood 1969). A special apparatus was constructed to automate this process (Pawley & Dole 1976). The advantage of this technique is that the sample is removed from the liquid phase without having a meniscus pass through it which could produce surface tension artifacts.

The dry blocks are then carefully mounted on SEM sample stubs with conductive paint, making sure to get the fractured surface uppermost. The conductive coating of gold-palladium is applied by sputtering in argon, and the sample is ready for viewing. The coating has the further advantage of a much greater secondary electron coefficient than organic tissue, enhancing the visibility of fine detail. The coating is perhaps 10 to 20 millimicrons thick, and varies with the local geometry. At the magnifications used in this study this does not obscure any of the structures of interest; microvilli on the type 2 cells are about 100 to 110 millimicrons in diameter.
RESULTS

Normal Tissue

Plate 1A is an illustration of some of the characteristic features of lung morphology that can be seen with the scanning electron microscope. This sample is a small piece from near the edge of a lobe; the pleural membrane can be seen on both sides. The hollow structure across the middle is a small airway, lined with ciliated epithelium, which looks rough at this magnification. Branching off towards the top is a terminal airway. Here the ciliated epithelium gives way to the smooth lining typical of the alveoli, which are seen as the small spherical cavities on the sides of the terminal bronchiole. Plate 1C is a detail of this transition region. The bulk of the sample is made up of alveoli cut in various planes, so that it is not always clear onto which terminal bronchiole they face. The solid structure just below the airway and the one in contact with it at the upper left are blood vessels. In this preparation the plasma is fixed in situ and appears solid. The tiny holes on the walls of the alveoli are the pores of Kohn, which presumably allow for some collateral ventilation from one alveolus to the next. Plate 1B is the other side of the same fracture, showing the other half of the same structures.

A series of micrographs at increasing magnifications shows more of the fine detail. Plate 2A, which is an area at the upper left of plate 1A, a dozen or so alveoli can be seen. The pores of Kohn are clearly evident, and the complexity of the structure may be appreciated.
At the lower edge, the erythrocytes in the blood vessel can be seen in the matrix of plasma. The pleura can be seen at the upper left; it was slightly torn by the forceps during mounting. In the next two plates, a type 2 cell becomes visible slightly to the right of center (plates 2B and 2C). In plate 2D the alveolar wall shows the details of the sutures between type 1 cells, and the microvilli of the type 2. Plate 2E shows the type 2 cell in greatest detail. The smooth area in the center is probably fixed surfactant, with a few microvilli showing through. The slightly coiled rod-like structure at the lower edge is probably contamination, but the type 2 cell does possess a single flagellum during embryogenesis. The last plate in this series, 2F, shows the microvilli in greater detail.

**Hyperplasia**

In the course of these investigations only two or three areas of possible hyperplasticity were found. This is largely due to the fact that these areas are not readily recognized at the dissecting microscope level, and at the scanning level there is too much "territory" to cover, whereas the tumors can be easily seen with a dissecting microscope and isolated for further investigation. Two of these areas are shown in plates 3 and 4. These are both razor cut samples of whole lobes, so the advantages of the freeze fracture technique do not apply.

Plate 3A shows an area of excess type 2 cells near the edge of a lobe (the pleura is seen at the lower edge). This cannot be positively identified as a true hyperplastic area since there is always a chance
that this many type 2 cells could come together randomly. This sample was not post-fixed in osmium, so the lamellar bodies are extracted; on the right hand edge, near the center, there is a type 2 cell that has been torn in half, showing the tiny pores that probably correspond to these organelles. Plate 3B shows a detail of 3A. The type 2 cells can be recognized by the microvilli and the white tufts of fixed surfactant on their surface. This sample is from a lung 28 days after urethane exposure.

Plate 4 shows a more developed hyperplastic area. This is from the same 28 day sample as plate 3, but is a different area. In the upper right quadrant of plate 4A somewhat thickened alveolar walls and an excess of type 2 cells can be seen. The torn edges of the razor cut obscure the wall thickening (compare with the tumor samples later). Plate 4B is a detail of this area; it is unmistakably unusual. Plate 4C is a monocular view of the area below plate 4B (rotated 90° counterclockwise) showing how far it extends.

In the course of examining large areas of lung with the scanning microscope no other areas of unusual thickening of alveolar walls, or other signs of hyperplasia of any sort, were found (see plate 19 for a possible exception). This lends credence to the idea that Shimkin and Polissar's hyperplastic areas actually were, at least partly, type 2 cells.

Tumors

A spontaneous tumor of the alveolar type 2 cell was found in an older mouse being used for another purpose. This is illustrated in
Plate 5. Plate 5A shows a quadrant of the tumor. It is a very dense mass, but the pleura (the upper left edge of the block) lies completely flat over it. There is no evidence for any appreciable compression of the surrounding tissue. Plate 5B shows a portion at greater magnification. Note how smooth (and featureless) the fracture plane is where it passes through the dense tissue. These areas in transmission microscopy show the dense columnar cell structure characteristic of adenomas. The small spaces in the dense mass can be seen to widen out at the tumor's edge into the alveolar and bronchiolar spaces of the surrounding normal tissue. Higher magnification showed the tumor to be of the usual type 2 cells.

The remaining tumors seen here are all from urethane treated tissues. The transmission micrographs that follow illustrate some of the features of tumor organization as well as the profile of the typical type 2 cell. Plate 6 is a good illustration of this. The alveolar air space is on the right, and the basement membrane (the dark fibers) is on the left. The large cell body is a type 2 cell, as can be seen from the light vacuoles that are the partly extracted lamellar bodies. The Golgi apparatus can be seen just above the nucleus and also on the left edge of the cell. The dark bodies are the mitochondria. The rest of the cytoplasm is filled with rough endoplasmic reticulum and ribosomes. The overall picture is of an energetic secretory cell. The type 2 cells can clearly be seen to form tight junctions (desmosomes) with the neighboring cells. There is also some evidence for attachment to the basement membrane. One very interesting feature illustrated here, and seen in most of the
tumor samples in both transmission and scanning, is the way the type 1 cell tries to cover the type 2, even in dense tumors. The lighter flap covering the type 2 at the right edge is a type 1. Also note the small whorl of surfactant in the alveolar space at the upper left.

Plate 7 is another transmission micrograph from the same tumor (these are all from a 124 days post urethane sample). Another cell type, the alveolar macrophage, can be seen at the upper right. Note that it makes very close contact with the type 2 cell's microvilli. The alveolar macrophage may serve to scavenge excess surfactant, which could attract it to tumor areas (the tumor cells continue to produce surfactant). A progression of lamellar bodies can be seen coming up the type 2 cell in the center. Other features of interest are the dense contacts with the fiberous structures at the lower right, the strange inclusions in the cell at the right border, which resemble curved cracks, and the small type 1 cover on the left-most cell. Also, a great variety of transition forms between the multivesicular bodies and the lamellar bodies may be traced in this micrograph.

Plate 8 shows many of the same features of plates 6 and 7. There is a strange thread-like projection of a macrophage coming down from the top. This is probably a thin flap cut in section.

The next series of SEM micrographs show the appearance of typical urethane tumors. Plate 9A is a small, not very dense tumor just under the pleura. This sample is 84 days post urethane. Again, there is no evidence of compression; the tumor simply spreads into the surrounding tissue. Plate 9B is a detail of the tumor itself. The rosette-like structure in the upper left is a blood vessel; the
erythrocytes can be more clearly seen in the next plate (9C). This is at the boundary of the tumor. Notice that the type 2 cells seem to be invading the normal tissue on the left. The ball-like objects about 8 microns across are probably alveolar macrophages. These seem to come in two forms; one is the ball-like structure seen here, and the other is a flat extended form. Plate 9D shows the detail of the tumor boundary at high magnification. The microvilli covered cells that are bulging out are type 2's, as can be seen from the vacuoles that are exposed where the fracture plane passes through them. Note how the type 2's seem to be stretched out, as though they were in motion. Plate 10 is a transmission micrograph of a possibly similar situation. This is from a different tumor of the same lung as plates 6-8. Here there are three type 2 cells along one side of an alveolar wall. Curiously, there are no lamellar bodies in this section, though the microvilli identify these as type 2's. Plate 11 is a section from the same tumor, showing type 2 cells on both sides of an alveolar wall. There is a wealth of structure here.

This plate clearly shows a blood vessel, probably a capillary containing an erythrocyte. An endothelial cell nucleus is seen just above the erythrocyte, and the characteristic endothelial lining extends around the vessel and off the plate on the left, where it has probably been cut at a grazing incidence. There are two type 2 cells in the upper part of the micrograph. The one on the right shows good examples of transition forms from multivesicular bodies to lamellar bodies. The one on the left is unusual in that bizarre tubular inclusions, "sequestrons", are seen along with the usual cytoplasmic
elements. One of these contains a mitochondria. Structures like this are sometimes found in more advanced tumors (see Brooks 1968, 1970). There is a thin flap of a type 1 cell covering the type 2 at the lower right.

Plate 12A is another small 84 day post urethane tumor much like that in plate 9. Notice the airway and its associated blood vessel at the lower right portion of the block. Plate 12B is a detail of the transition region at the edge of the tumor.

Plate 13 shows a larger, slightly denser tumor from the same sample. Note that there is no evidence of compression again. The blob at the lower edge of the block is silver paint that was inadvertently put there during the mounting process.

Plate 14A shows a larger, denser tumor, again from the same sample, more like the spontaneous tumor in plate 5. There is still no evidence of compression, just simple invasiveness. Plate 14B is the other side of the same fracture, except that a few more flakes have come off during the fracturing process. 14C is a detail of the boundary, showing the transition from tumor to normal tissue. Notice that the tumors are more dense in the central areas, and less dense near the edges, and then merge into the normal tissue. Plate 14D shows the very edge of the transition area. There is an abundance of macrophages, mostly of the spherical form, but transition forms to the flatter kind can be seen.
Panorama

To better illustrate the transition from the dense tumor mass to the surrounding tissue, a series of overlapping micrographs was made at fairly high magnification on a small 124 day tumor. This is illustrated in plates 15 and 16. Plate 15A is a view of the whole block with the tumor embedded in it. 15B and C are higher magnification views of the tumor area. The area scanned in the series 15D, E, and F is seen in the upper center of 15B, alongside the airway. The lower portion of the scan, 15E and F, can be seen in 15C. The actual panorama, 15D, E, and F, is mounted two stereo pairs to a page (D and E, E and F) so that the transition can be seen more easily.

Plates 16A through E are the opposite side of the fracture in the same tumor. In fact, 15F and 16C are the same area, but rotated 90°. The scan 16C through E is in a different orientation than the larger views A and B; it crosses the center of B and goes off to the left. In studying these micrographs, note the various forms of macrophages and the way the type 1 cells maintain a covering over the mass of type 2's.

Oddities

The preceding micrographs have illustrated the usual sort of tumor produced by urethane exposure. One example of another morphology was seen in a razor cut section of a 124 day sample, illustrated in plates 17A and B. This tumor shows a very definite tendency to compress the surrounding tissue and to raise the pleura. It seems to grow in the air space and compress the tissue, whereas the usual picture is
to grow in the alveolar walls and compress the air space. Plate 17B is a monocular view of a portion of this tumor; it is clearly comprised of type 2 cells, yet it shows a very different pattern. This may correspond to the more sarcomatous tumor type that is sometimes seen in this strain.

To contrast the tumor seen in these micrographs, plates 18A–C show a normal type of tumor also cut with a razor. Although it is not so clear as with the freeze fracture samples, there is no appreciable compression of the surrounding tissues, nor is the pleura raised by the tumor mass. Plate 18B shows the transition area to normal tissue. Note that even where the alveoli have been severely restricted by the thickening mass, there is still usually a normal type 1 epithelial covering. The one area where this is not the case is shown in greater detail in plate 18C. Here the epithelium is clearly the ciliated type 2 cells. This sample was not post-fixed in osmium, so the lamellar bodies can be seen in the torn type 2 cells as tiny pores.

The last series of plates show what may be an immune response to an area of hyperplasia. 19A shows an overview of the area. It is only a few alveoli across, just a little below the pleura. 19B is a closeup of the area; there is clearly some unusual activity, especially in the right portion of the micrograph. The three following plates, 19C through E, are a scan across this area from left to right (these were taken on a different microscope than the others, so the resolution is not so good). 19C shows that there is an excess of type 2 cells as well as macrophages. The large bright object in the
center is probably a type 2 cell; the macrophages are the flat objects at the upper right. 19D is immediately to the right. There are a large number of macrophages "attacking" something. The type 2 cells can be identified by the pores in the cytoplasm where they are cut, and by the microvilli. Plate 19E is just to the right of D. Note how flat the macrophages are here (one can be seen on edge in the space at the upper right). While this may be an immune rejection of a nascent tumor, it may be other things as well, such as a focal infection, with the excess type 2 cells merely being a nonspecific response to injury.

Perspective

The overall picture of these tumors is one of well behaved invasive growth. The growing tumor does not disrupt the basic structure of the lung. Rather, the tumor cells move within the alveolar walls, maintaining the normal relation to the other cell types there, and then gradually thickening the walls and narrowing down the alveolar space until the tumor becomes dense, at which point there is no further growth. Almost everywhere the type 1 cells cover over most of the mass. The unique perspective afforded by the scanning electron microscope makes these new interpretations possible.

The individual cells must have some capacity for motion. They migrate from the dense central areas into the surrounding tissue where they multiply and fill in the spaces. This motility is the crux of this work, its thesis. The following sections discuss the consequences of this using a simple mathematical model.
Plate 1b. 58x
Plate 2b. 595x
Plate 2c. 1,160x
Plate 2e. 5,780x
Plate 2f. 12,700x
Plate 3a. 760x
Plate 4c. 1,210x
Plate 5a. 110x
Plate 5b. 230x
Plate 9a. 100x
Plate 9b. 245x
Plate 9c. 475x
Plate 9d. 2,340x
Plate 11. 9,460x
Plate 12a. 92x
Plate 13. 100x
Plate 14b.  86x
Plate 14d. 950x
Plate 15a. 72x
Plate 16b. 325x
Plates 16c & 16d. 680x
Plates 16d & 16e. 680x
Plate 17b. 530x
Plate 18a. 77x
Plate 18b. 380x
Plate 18c. 1,540x
Plate 19b. 500x
Plate 19c. 1,450x
Plate 19d. 1,420x
Plate 19e. 1,450x
Consequences of Motility

If the type 2 cells move, what governs their motion? How fast and which way do they go? All cells possess the rudimentary apparatus for motion in the microskeletal components of microtubules and microfilaments, and these cells in particular also have anchor points with which to push and pull in the desmosomes and attachments to the mesenchymal fibrous network. In general, one would not think of epithelial cells as being motile, but, for example, they would have to be for wound healing to occur. The type 1 cells are probably not (as) motile since they are so extended and lack much in the way of cytoplasmic structure. But the type 2 cells, being the type 1 precursor, would have an advantage in being motile since they could then keep a fairly uniform distribution throughout the lung and not be as subject to local depletions. For the purposes of this discussion, the simplest assumption is made about this motion: it is completely random.

The assumptions of this model are simple and modest. A more detailed set of assumptions are of course possible, and could perhaps give a more detailed description of the system, but those used here are sufficient. The features of the alveolar tumor system that the resultant model is able to describe are: (1) the time lag after urethane exposure to the first appearance of tumors, without prior clonal aggregations, (2) the linear relation of tumor radius with time,
(3) the square root of time dependance of the radii of the hyperplastic areas, (4) the even proportional growth of all tumors, and (5) the density profile across the tumors.

Random Motion

The first assumption is that the type 2 cells normally move at random throughout the volume of the lung. In actuality, the motion is restricted to the alveolar walls, so it is more like a binomial distribution, but on the size scale of tumor growth (and by the central limit theorem) this is nearly the same as if they were true Brownian particles in a homogenous volume. Random motion is characterized by an average "step size" per unit time (see Feynman et al. 1964); the root-mean-square step length gives the correct kind of average. The step size will be symbolized as $S$ in the derivations that follow. It is quite likely, and probably necessary, that $S$ varies from one cell line to another in a random way.

The probability density distribution of a randomly moving particle after a certain number of steps can be described by a three dimensional Gaussian,

$$
\frac{1}{(\sqrt{2\pi}\sigma)^3} e^{-x^2/2\sigma^2}
$$

Here $x$ is the distance the particle has travelled from the origin in the course of the walk, and the standard deviation $\sigma$ is the width of the distribution at $1/e$ of its maximum value. $\sigma^2$ is the mean square step size times the number of steps taken, $S^2t$. The number of steps taken is a dimensionless quantity numerically equal to time; it is
symbolized t. Thus the density distribution of a single particle expands to
\[
\left( \frac{1}{\sqrt{2\pi} \sqrt{t}} \right)^3 e^{-x^2/2s^2 \tau}.
\]
(2)

It will be more convenient to refer to this function simply as \(P(x,t,s)\).

This function has a width \(\sigma\) that increases as the square root of time. If it is assumed that the hyperplastic areas seen by Shimkin and Polissar (1955) are pre-tumor "clones" of transformed cells that are not yet dense enough to interfere with the randomness of the cells' motion, the excellent fit of their radii by a linear function in the square root of time (see figure 2) is very encouraging. The average radius fits the data with such a function up to 15 weeks with a coefficient of determination \(r^2\) of .95, and the largest radius fits up to 12 weeks with an \(r^2\) of .99.

Some difficulties arise however. During the period mentioned above tumors are steadily appearing. So either the hyperplastic areas are becoming tumors and new areas are appearing, or the hyperplastic areas are merely another effect of the urethane unrelated to the tumors. The latter possibility could be a serious objection, especially since Shimkin and Polissar did not identify cell types. However, in the course of examining razor cut lung slices of whole lobes a definitely hyperplastic area of type 2 cells was found (see the previous section on "hyperplasia"). At least some of the hyperplastic areas are of type 2 cells. The former objection can be considered like this: rather than a hyperplastic area merely growing slowly with time, it will "fill in" and become a tumor, and no longer be counted as a
hyperplastic area. Thus at each sample time a different population of hyperplastic areas is measured. This could throw everything off if early appearing areas were somehow intrinsically different from late appearing areas. If a clone's behavior can be purely described by its cells' step size and rate of division, then an area would become visible when its central region reaches some critical density (which would have to happen eventually since exponential increase is faster in the long run than square root cubed decrease), and at that time its size would be a pure function of the square root of time and $S$, but only if the step size and doubling time are not statistically correlated. If they were correlated very strongly, the excellent fit of the data to the square root of time would be fortuitous. In a later section evidence is educed that there may not be a strong correlation.

Growth

To explore the idea that the behavior of a clone of cells is purely described by two parameters, their step size $S$ and doubling time $T$, let the total number of cells derived from a single transformed cell as a function of time be represented by $N(t,T)$, where $T$ is the doubling time and $t$ the elapsed time since the initial transformation. (The same symbol is being used as for the unit of time used in the step size considerations since they are numerically equal.) The most reasonable form to take for $N$ is simple exponential increase:

$$N(t,T) = 2^{(t/T)} = e^{(t \ln 2 / T)}.$$  (3)
This assumes that there is no constraint on growth. Then the two functions \( P \) and \( N \) can be simply multiplied together to give the density of cells at a distance \( x \) at time \( t \):

\[
D = N(t,T) \cdot P(x,t,S)
\]  

which expands to:

\[
D = \left[ \frac{1}{(S\sqrt{2\pi}t)^3} \right] e^{(t \cdot \ln 2/T - x^2/2 \cdot S^2 t)}
\]

This represents a Gaussian distribution that is increasing in both width and height.

**No Growth**

Since it is known that the tumors are very "well behaved", that is they do not reach infinite density, nor even exert any appreciable growth pressure on the surrounding tissue, it will be assumed that the type 2 cell responds to some sort of cell contact mediated inhibition of growth. This is clearly not the mechanism that controls their population size in the normal state since they are usually solitary. They are capable of very rapid increase, as is demonstrated if the type 1 cell population is injured (say by an oxidant such as ozone or nitrogen dioxide), so there is some sort of finely tuned mechanism to control their numbers. The next assumption is that most of the type 2 cells will respond to cell contact inhibition of growth, even though this mechanism is never exercised in the normal state, but that the primary event of transformation is a release from the normal mechanism.
In the derivations that follow, some sort of growth inhibition will have to be assumed to prevent the density from achieving arbitrarily large values. The approach that will be followed is simply to apply some limiting function to the density that would result if there were no inhibition. The exact form of this function is not critical, so long as it is monotonic and continuous, approximates the uninhibited value of the cell density \( D \) for small \( D \), and some limiting value, say \( A \), for large \( D \). This limited density is \( d \):

\[
d(t,x,T,S,A) = H_A(D) = H_A(N(t,T) \cdot P(x,t,S)),
\]

where the form chosen for \( H \) in the calculations is simply

\[
H_A(D) = \frac{D \cdot A}{(D + A)}.
\]

This is a variation of "\( x \) over one plus \( x \)" that approaches \( D \) for small \( D \) and \( A \) for large \( D \). Other forms are certainly possible, but the choice is not critical. \( A \) is taken as a constant in these derivations, usually with a value of .001 cells per cubic micron.

This approach is only a first approximation; its deficiencies are one, that it will overestimate the diffusion from the central areas because the density limitation is applied after the outward diffusion is calculated, and two, that it will overestimate inward diffusion since the cells are assumed to be point particles that can freely diffuse without regard for steric considerations. The two effects may in fact cancel, but most of the results are derived for contours of constant density, which by the nature of the monotonicity of the density limitation will remain constant under any simple (local)
density limitation function.

A more complete approach would be to do a discrete numerical simulation with a computer, and that should probably be the next step in analyzing this problem, but the simple analytic methods used here are surprisingly good.

Features of the Model

Figure 4 shows the result for solving for d as a function of distance from the tumor center for various values of time. Five sets of values for S and T are used to illustrate the effects of reasonable variation. The various values for the parameters are chosen as follows: The limiting density A is always taken as .001 since a cell is about 10 microns on a side, so there are about 1000 cubic microns in a cell. The time t varies from 10 to 140 days, which is the range studied by Shimkin and Polissar. The radius x is typically between zero and a few hundred microns (these are small tumors at this stage). The two parameters, S and T, are allowed to vary in the ranges of 2 to 5 days for the doubling time, and 2 to 5 microns per day for the step size (see following section "On Estimating S and T"). The units of the density d in these graphs are in percent of the maximum density A, so the values will range from 0% to 100%.

An examination of the graphs reveals some interesting features. At the larger values of time the contours tend to be evenly spaced; that is, the radius of the tumor increases linearly in time at sufficiently large time. This is very significant. Another feature is that, except for small values of both S and T, there is a time lag
Figure 4. Solutions of equation 6 for various values of the parameters step size $S$ and doubling time $T$ as a function of time and distance. The vertical axis of each of the five plots is the tumor density, ranging from 0 to 100%. The horizontal axis is radius from the tumor center in microns. The families of curves represent the tumor density along the transect through the tumor center.

The most obvious feature is that the tumors begin to grow at a linear rate once the central area reaches its maximum density. The other feature is that it takes a while before the tumor becomes sufficiently dense to be noticed. The curves all display a sigmoidal character at the tumor "boundary", with the shapest boundary in case C with the shortest step size and the shortest doubling time. The least distinct boundary is found in B with the greatest step size and doubling time. The fastest growth is case A with the largest step size and the shortest doubling time. Case D is the opposite.

The values chosen for the parameters represent probable values estimated from figure 6. They do not include the full range of possibilities, but rather are intended to be typical. Case E is chosen to be the most typical value.
Figure 4.
before the central density reaches an appreciable value; the central density increases to its limiting value sigmoidally. The density is also sigmoidal at the edges of the tumor. It can be seen that the width of the region of intermediate densities at the tumor's edge is constant once the tumor has entered into the linear growth phase.

**Linear Growth**

Contours of constant density $d(x,t)$ will also be contours of constant density in the uncorrected density $D(x,t)$. Solving the expression of $D$ in terms of $x$, $t$, $S$, and $T$ (equation 5) for $x$ as a function of $t$, $S$, $T$, and $D$ gives

$$x = \sqrt{2S^2 \left( \frac{t \ln 2}{T} - \frac{3}{2} \ln t - 3 \ln S - \ln D - \frac{3}{2} \ln (2\pi) \right)} \quad . \quad (8)$$

For large values of $t$ (say greater than 100 days) the dominant terms become

$$x \approx \sqrt{\left( \frac{2S^2 t^2 \ln 2}{T} \right)} \approx 1.175 \frac{S t}{\sqrt{T}} \quad , \quad (9)$$

provided $S$ and $T$ are not too large. Thus the contours of constant density in terms of tumor radius and time will be linear in time and step size, and inversely related to the square root of the doubling time. This means that the radial growth rate $dx/dt$ will be fairly insensitive to variations in cell doubling time; if the doubling time for one tumor cell line is ten times that of another, it will grow only three, rather than ten, times more slowly ($[2^{10}]^{1/3} = 10$). The step size is a much more critical parameter for determining tumor
growth rate. Another result is that the size distribution of a population of tumors will maintain a constant proportionality; Shimkin and Polissar (1955) note that in their data there is a fairly constant order of magnitude spread in the tumor population cross sectional areas (i.e., about a factor of three in radius) as the tumors get older.

Figure 5 is a graph of equation 8 for various values of $S$ and $T$. The curves represent the tumor radii (50% density) as a function of time. The most striking feature is the linear growth once the tumors have formed. It is clear from examining these curves that the approximation of equation 9 holds best for small values of $S$ and $T$ (curve C) and less well for larger values (curve B). It can be seen how the tumor population as a whole maintains a fairly constant ratio of largest to smallest tumors through time. Note the distribution of times until tumor "appearance".

**Estimating $S$ and $T$**

The probable range and most likely values for the step size and generation time can be estimated. The smallest possible value for the generation time $T$ is the cell cycle time, probably not much less than the 45 hour value derived by Dyson and Heppleston (1976). This is represented in figure 6 (which is a plot of $S$ against $T$) as the vertical boundary on the left. Consideration of equation 9 permits further restrictions to be derived. This formula relates the ratio of tumor size $x$ to time $t$ for given $S$ and $T$. This ratio can be found from Shimkin and Polissar's data (1955). Averaging the largest ratios
Figure 5. Solutions of equation 8 for various values of the parameters step size $S$ and doubling time $T$ as functions of time and tumor radius. The curves represent solutions for a 50% density. The tumors clearly grow at a linear rate once they get started. The $\circ$ marks are the data points from figure 2. It is clear the averages are taken from a changing population. The parameter values chosen predict too small a size in the early stages of growth, in the 30 day area, but illustrate the general features of the pattern. Although they get started at widely different times, the three curves with equal values for $S$ and $T$ (B,C, and E) grow at about the same rate, while the fastest and slowest curves have complimentary values for $S$ and $T$. The slope of the curves is most strongly governed by the step size, with the doubling time having a lesser effect; curves A and B are in one family, while C and D in another. The greater step size for A and B causes them to appear later, but grow faster than C and D. E is an intermediate case.
Figure 6. This figure shows the limitations imposed on the combinations of $S$ and $T$ by the model when applied to Shimkin and Polissar's data. The minimum possible value for $T$ is the cell cycle time, taken from Dyson and Heppleston as 45 hours (1.88 days). Examining equation 8, which is tumor radius as a function of the other variables, gives a series of relations for $S$ and $T$ if the radius, time, and density are appropriately chosen, that can be solved numerically giving the curves A, B, and C. Possible values of $S$ and $T$ will lie between the sets of curves generated by the maximum and the minimum tumor sizes. Equation 5 can be rearranged and solved for $x=0$ at the time of the last appearing tumors (50 days) yielding equation 10, which is the decaying exponential on the right side of the plot. This then bounds an area that will contain all reasonable values for $S$ and $T$. This is sketched in with the free-hand oval. The illustrative values used in figures 4 and 5 are taken from this area. Taking different values for the density $A$ in equation 7 will change some of the details of this plot, but this serves to illustrate that the model's parameters can be estimated.
Figure 6.
together gives about 3.0 microns per day for \( S/\sqrt{T} \), and the smallest 0.8.

These two conditions are plotted as the horizontal parabolas labelled A in the figure. The exact equation (5 or 8) for \( S \) as a function of \( T \) involves the time \( t \) in an explicit manner and cannot be solved analytically. However, numerical root-finding methods yield the curves B and C for times of 200 and 100 days. The curves are of an obviously different form, but do not diverge too much in the region of interest. The last boundary can be derived from considering the time of appearance of the tumors. Assuming that a tumor "appears" when its central density reaches some critical value (say 50%), equation 5 may be rearranged for \( S \) as a function of the other parameters (letting \( x = 0 \)) yielding an exponential:

\[
S = \frac{1}{3^2/2^{3T}} e^{(t \cdot \ln 2/3T)}
\]  

(10)

Solving this for \( D = 0.001 \) (i.e., \( d = 50\% \)) and using Shimkin and Polissar's value of 50 days for the latest appearing tumors, the curve plotted on the right results. Using a value of 30 days, which is the median time for tumor appearance, another similar curve results that can be used to estimate probable values for \( S \) and \( T \). The area bounded by the cell cycle time, the smallest tumors, the latest tumors, and the largest tumors, within which a freehand oval has been sketched, represents the likely limits for combinations of \( S \) and \( T \). The smaller oval in the center, where the curves for the average sized tumors (lines A, B, and C) and the median time of appearance (30 days) intersect shows the probable median values for \( S \) and \( T \). The limiting
area shows no pronounced tendency for skewedness, so S and T are probably reasonably independent.

To sum up, the step size is found to range from about 2 to 5.5 microns per day, with the most likely value being about 4 (a little less than half a cell diameter), and the generation time from about 2 to 6 days, having a most likely value of about 4 days. These values seem to be physiologically reasonable, but it must be remembered that they are contingent on the particular form of the model.
SUMMING UP

The Model

From a simple model it has been possible to describe most of the growth and form of the urethane induced alveologenic tumor in the strain A mouse and to estimate the values of the model's parameters from existing data. The model is inspired by the idea--gleaned from examining these tumors with the scanning electron microscope--that the type 2 cells that form this tumor are motile and the assumption that this motility may be simply expressed as random motion. A few further assumptions were made to aid the analysis, such as exponential increase in the absence of crowding, and contact mediated inhibition of growth in crowded conditions. It works quite well.

Random motion can be described using the classical concept of the random walk, wherein the probability density function is a three dimensional Gaussian ("bell-shaped-curve") that gets wider as the square root of time and flatter as the cube of one over the square root of time. The parameter that describes the rate of change of this function is the root-mean-square step size per unit time. The transformed cell is also multiplying exponentially with a characteristic doubling time. Putting these together gives the basic features of the model which is then described by the two parameters. Most of the interesting results need no further assumptions than this, but physically there is a requirement that growth be inhibited in the central regions of the tumor, as is clearly seen in the micrographs,
when the cell density reaches a certain point. None of the results depend critically on the form this inhibition takes, so a simple function is chosen for illustration. This simple model successfully predicts (1) the square root of time dependance of the size of the presumably pre-tumor hyperplastic foci, (2) the linear growth of tumor radius with time, (3) the time lag until the appearance of the tumors, (4) the density profile of the tumors, and (5) the distribution of tumor sizes through time. The model is not complete, since the problems of migration of cells into and out of regions of high density are not dealt with adequately, nor are the details of growth inhibition in the dense areas. These effects may to some degree fortuitously cancel.

What Happens

A scenario of the formation and growth of a tumor may be something like this: Prior to the urethane exposure, the type 2 cells of the lung epithelium migrate about in a random manner, maintaining a fairly even distribution, secreting surfactant and replacing the type 1 cells as the need arises. The urethane exposure is of brief duration; some of the cells are in a critical phase of their cell cycle whereby the urethane, a potent DNA antimetabolite, damages the genome in some manner so that they are no longer able to control their rate of division by the normal mechanism (which is currently unknown). The cells that are so transformed represent a variety of migration and reproductive rates. As one of these transformed cells migrates and divides, its daughters populate a slowly growing region of the lung, which is as yet indistinguishable from any other area. This "seeded"
area is in an exponential growth phase since there is relatively little contact between the transformed cells. As the area grows the center fills in and eventually becomes solid. Thus the tumor appears some time after the event of transformation, even though it has been growing the whole time. Depending on the various growth and migration rates this happens sooner or later and results in a larger or smaller tumor. The edges of the tumor continue to expand outward, invading the surrounding tissue and then filling in after. As the tumor becomes larger, this proceeds at a linear rate. In the central areas, as soon as the tissue is fairly solid, growth stops. This may be due to a vestigial cell contact mediated inhibitory mechanism left over from some earlier embryonic cell type, yet not damaged by the urethane exposure. The central area takes on an even, well structured appearance, with the alveoli and bronchioles being squeezed down to appear like ducts in a glandular tissue. The important thing to keep in mind about these tumors is that the event of transformation is not a release from cell contact mediated inhibition of growth, as is probably the case in most other types of solid tumors, but a release from some other (unknown) mechanism of inhibition that keeps the type 2 cell population at its usual sparse level, with cell contact inhibition remaining intact only as an embryonic vestigial capability. Thus there is little tendency for metastasis; what malignancy is seen is probably due to the vastly increased population of type 2 cells in the tumors (which are of an already damaged genome due to the urethane exposure) simply representing a higher risk of a further
single event that could release a cell from contact inhibition as well. The few tumors of the other structural type probably represent a release from both mechanisms at an early stage. There are, however, a few unanswered questions.

Remaining Problems

The hyperplastic foci described by Shimkin and Polissar are presumably precursors to the tumors, but during the course of tumor formation there may be over 600 such foci in the whole lung, whereas only some 35 or 40 tumors eventually appear. What happens to the rest of them? Since Shimkin and Polissar did not identify cell types, they may represent something completely different. On the other hand, the facts that these foci reach a maximum number some 5 or 6 weeks after the urethane exposure, and that some hyperplastic areas of type 2 cells are seen with the scanning electron microscope, argues that at least some of them are composed of type 2 cells. So the other possibility is that most of the foci represent cell lines with immunologically detectable cell surface antigens, and that the animal's immune system simply takes care of them. The reason the strain A mouse is so prone to the urethane induced tumors is that it possesses the tumor associated surface antigens for this tumor as a normal tissue antigen, so that the immune system does not recognize (at least some of) the tumors. It may well be that there are other possible urethane induced changes in the cell that release it from the normal growth control mechanisms, producing recognizable surface antigens as well, and the excess hyperplastic foci may represent these. The large numbers of
macrophages seen in some areas may be the consequence of this recognition, as in plates 19A-E.

If it is the case that all or most of the hyperplastic foci represent pre-tumor conditions, but that most of them are rejected by the host's immune system, the administration of urethane to a resistant strain of mouse could produce the same hyperplastic foci, but reject all of them. This would be fairly easy to test.

Other problems remain with the model's assumptions, as were discussed earlier. To study the system with a more accurate model would require a numerical approach, rather than the analytic one used here. More parameters would probably be introduced, making it more difficult to estimate them from experimental data.

One approach would be to do a series of simulations based on the idea of following all the cells arising from a single transformed cell, taking into account steric considerations. The problem of contact inhibition would have to be dealt with in a more careful way. Another idea would be to divide the volume around the original cell into shells of uniform thickness, and allow the cells in a given shell to migrate in either direction as well as multiply. There would still be the two parameters of mobility and intrinsic rate of increase, as well as those of steric hinderance and contact inhibition.
MATERIALS AND METHODS

Animals

Female A-jax mice from the Jackson Laboratories, Bar Harbor, Maine, were used in this study. They were all about 10 weeks old at the time of the urethane injections, and weighted about 20g. They were housed in the animal care facilities at the Lawrence Berkeley Laboratories for the duration of the experiments, under standard conditions. To reduce the incidence of respiratory disease, they received terramycin in their drinking water the first 4 or 5 days after their arrival, and 10 ppm chlorine thereafter. A single dose of urethane was used, 0.75 mg/g intraperitoneally, dissolved in distilled water. Control animals received distilled water.

Tissue

The animals were heavily anesthetized with sodium pentobarbital. A small incision was made in the trachea for the later instillation of fixative. The thorax was opened via the abdomen, allowing the lungs to collapse. The descending aorta was transected in the abdomen to reduce blood pressure so that the lungs would not be congested during fixation. Then the fixative was introduced into the trachea under a pressure of about 30cm-H₂O. During the first few moments slightly more pressure was applied to start the filling process. The lungs were allowed to stand in the thoracic cavity for at least an hour under pressure with the fixative. Then they were removed and the trachea tied off with the lungs in the inflated state and stored in
fixative overnight. The lobes were then separated and stored in cacodylate buffer. The ultrastructure seemed to be stable in this condition for at least 6 months.

Fixative

The standard fixative used was a variation of Karnovsky's solution after Jan Nowell (personal communication). 0.2M cacodylate buffer at pH 7.2 was used as the stock buffer throughout. To make the stock fixative solution, dissolve 4g paraformaldehyde powder in 50 ml H₂O with 30 ml stock buffer by heating to 70°C and adding a few drops NaOH. Cool, and add 10 ml 50% gluteraldehyde, 0.05g CaCl₂, and 10 ml stock buffer. Dilute 4.5 : 1 by adding 100 ml H₂O and 350 ml stock buffer to 100 ml of the stock fixative. Adjust to pH 7.2 with 1N HCl, store cold, and filter before use. It should be refreshed every week or two. This results in a 550 milliosmolar fixative.

Processing

The fixed tissue in buffer stores well for many months at 4°C. The lobes were examined for tumors with a low power dissecting microscope. These, along with normal tissues, were cut from the rest of the tissue with a sharp xylene-cleaned razor blade as small blocks about 2x2x5mm, with the tumor in the center. These were usually post-fixed in 1% buffered osmium tetroxide. They were then rapidly dehydrated through a graded alcohol series (50%, 75%, 90%, 95%, 2x100%; 5 minutes each) after rinsing in distilled water to remove the buffer salts. Those samples for TEM examination were removed from the
process at this point (see below).

**Fracturing**

Humphreys' technique was used to cryofracture the blocks. They were placed in small tubes of Parafilm in alcohol and the ends crimped off. These were then plunged into liquid nitrogen. The alcohol freezes into a clear glass-like solid without crystal formation. These were fractured with a chilled razor blade on a small metal block still under the liquid nitrogen. The tissue and the osmium darkened tumor are still slightly visible in the Parafilm tube, and the fracture is placed as close to the tumor center as possible. The fractured blocks were thawed in alcohol.

**Drying & Mounting**

The fractured blocks were dried by the critical point method in a special apparatus built to automate the process (Pawley & Dole 1976). They were transferred to acetone from the alcohol in a graded series, and then dried with CO$_2$. The blocks were carefully examined with a dissecting microscope, and those with a fracture plane through or near a tumor were mounted on SEM sample stubs with conductive paint. A map of the stub was drawn with the individual blocks labeled. The stubs were then coated with a gold-palladium alloy by sputtering in a specially built apparatus. There was no vacuum gauge, but 900 volts at 22 milliAmperes were used, and the argon pressure was adjusted to give a 2-3 mm dark zone around the cathode. After 10 minutes the coating was about 10-20 millimicrons thick. Very little granulation is evident with this method, and it gives better coverage of the
complex surface of the lung than evaporation does.

**Scanning**

The stubs were examined at 10 kV, usually at 0° tilt, with an AMR 1000 scanning electron microscope. Pictures were taken on type 52 Polaroid film. Stereo views were taken by tilting the stage 5° between views and realigning the field of view. Plates 19A-E were taken with a JSM-l with Polaroid type 42 film.

**Transmission**

The tissue blocks for the transmission plates were trimmed and embedded in Araldite. Sections were cut with fresh glass knives and mounted on uncoated 400 mesh copper grids. They were post-stained with uranyl acetate and lead citrate, and viewed with an AEI-802 at 80 kV. Micrographs were taken on glass plates. Thick sections were also cut from the samples and mounted on glass slides and stained with toluidine blue for visual examination with an oil-immersion microscope.
REFERENCES


STEREO VIEWING

The basic problem in three-dimensional viewing of a stereo pair is to provide each eye with an in-focus, correctly aligned image, with appropriate parallax in the two images. In SEM work the parallax is provided by tilting the sample a small amount (5° was used in this work) for one of the micrographs. The two micrographs are reduced and mounted side by side so that the one taken at the greater tilt (in a clockwise sense) is on the left. They are mounted about 65 mm center-to-center, which is the average interpupillary separation. To view the pair, the eyes must diverge so that each looks at the appropriate image, and then focus on the plane of the images. This is normally difficult to do since the divergence and accommodation mechanisms of the eyes are coupled, but it can be learned with practice.

A simple optical device may be contrived to provide either the accommodation necessary for the pair's separation (by a pair of converging lenses) or the divergence for the viewing distance (with weak prisms) or a combination of both. Simple converging lens pairs in appropriate mounts may be purchased from suppliers of electron microscope or geodesy and surveying equipment (two are listed below). A simple device was constructed as part of this work for viewing the stereo pairs before they were reduced to the 65mm separation. It was based on the antique stereoscope. It consisted of two off-axis sections of a large achromatic convex lens (focal length 400mm, diameter 65mm) mounted so that the optical axis of each section was opposite the other. The two lenses had an adjustable separation.
(40 to 80mm) and were mounted so that they were about 300mm from the stereograph to be viewed (virtual image distance = 1,200mm).

A barrier was provided down the middle to prevent the eyes from looking at the wrong image. This was suitable for viewing the unreduced pairs (separation = 90mm) as well as the reduced ones. The achromatic lenses provided very sharp resolution without distortion or colored fringes.

Sources

Ernest F. Fullam Inc.
Schenectady, N.Y.

Gordon Enterprises
This report was prepared as an account of work sponsored by the United States Government. Neither the United States nor the United States Energy Research and Development Administration, nor any of their employees, nor any of their contractors, subcontractors, or their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness or usefulness of any information, apparatus, product or process disclosed, or represents that its use would not infringe privately owned rights.