RATES OF CELL DIVISION DURING EPIDERMAL AND PULMONARY CARCINOGENESIS

A Thesis

Presented to

Faculty of Graduate Studies and Research

The University of Manitoba

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

---

by

John Alexander Wallace

October 1964

THE UNIVERSITY OF MANITOBA ELIZABETH DAFOE LIBRARY

ABSTRACT

The rates of mitosis in preneoplastic and neoplastic epithelia of mouse skin and lung were determined by the colchicine technique.

Five experiments are reported.

In Experiment No. 1, the rates of ear and interscapular epidermis were ascertained during a 24-hour period following a single painting of benzpyrene. Thirty-four per cent fewer epidermal cells divided in treated than untreated ears. The inhibition of mitosis occurred 12 to 24 hours after treatment. The hair cycle influenced mitotic activity of the interscapular epidermal cells and it was not possible in this experiment to separate the carcinogens effect on mitosis from that of the hair cycle.

In Experiment No. 2, multiple paintings of benzpyrene were administered at weekly intervals to interscapular epidermis of two groups of mice. Mitotic rates for a six hour period were determined at weekly intervals. In one group, the mitotic rates of interfollicular epidermis indicated a proliferative pattern consisting of an initial lag period, a period of progressive cell increase, followed by an inhibition of cell division and a terminal recovery stage of increased mitosis. The significance of this pattern and its relationship to skin tumorigenesis is not known. In the other series

the mitotic rates of hair follicular epithelium were determined during the histogenesis and regression of Type 1 keratoacanthoma. The results suggested that the mitotic activity of the developing, mature and regressing Type 1 keratoacanthoma paralleled the mitotic activity of the hair follicle during its cycle of development, hair production and regression. Two subtypes of Type 2 keratoacanthoma were described, one subtype was similar in morphology and mitotic rate to a Type 1 keratoacanthoma, while the morphology and mitotic rate of the other approached that of an epidermoid carcinoma. The mitotic rate for a six-hour period of the epidermoid carcinoma was 16 per cent and the doubling time was estimated to be 1.6 days.

In experiment No. 3, two types of alveologenic neoplasms were observed. One of the 36 tumors was described as a papillary adenocarcinoma. The remaining tumors were classified as papillary adenomas. The daily mitotic rate of the papillary adenoma was 1.20 per cent which was approximately four times less that of normal alveolar tissue. The doubling time was estimated to be 83 days. A diurnal rhythm of mitosis was evident in the papillary adenoma. The papillary adenocarcinoma had a mitotic rate six times that of a papillary adenoma growing in the same lung.

In Experiment No. 4, adult mouse lung tissue was removed and impregnated in vitro with 1.3 mg. of urethane

crystals and transplanted to the subcutaneous tissue of homologous mice. Tumors were not observed at 77, 105, 212 and 343 days after implantation. Urethane apparently does not produce lung tumors when directly applied to grafted lung tissue.

In Experiment No. 5, the experimental method was similar to that of Experiment No. 4 except that each lung graft contained 0.2 mg. of methylcholanthrene crystals. One hundred nine days after implantation 18 tumors were recovered from 25 mice, one tumor from each mouse. There were nine epidermoid carcinomas, four fibrosarcomas, four undifferentiated carcinomas and one adenocarcinoma. The average six-hour mitotic rate of the epidermoid carcinomas was 5.11 per cent. The adenocarcinoma had a mitotic rate for a six-hour period of 2.06 per cent. The mitotic rates of the other tumors were not determined.

ACKNOWLEDGMENTS

The author wishes to thank Dr. F. D. Bertalanffy for his kind advice, criticism and encouragement during the preparation of this report.

To Dr. I. M. Thompson, Professor and Chairman, Department of Anatomy, the author wishes to express his appreciation for being extended the privilege of studying in the Department of Anatomy.

For the preparation of the tissues, the author is pleased to acknowledge the assistance of Mrs. R. Murray, Mrs. C. Simpson and Mrs. S. Kron.

The graphs were prepared by Mr. M. Stover, whose skill the author acknowledges.

The author wishes to express his appreciation to Mr. L. Chua for recording some of the mitotic counts.

This investigation was supported by a research grant to Dr. F. D. Bertalanffy from the National Cancer Institute of Canada.

TABLE OF CONTENTS

CHAPTER		PAGE
I.	INTRODUCTION	1
	Problems	1
	Importance of the study	2
II.	REVIEW OF THE LITERATURE	6
	Microscopic anatomy of mouse skin	6
	Epidermis	6
	Pilosebaceous apparatus	7
	Dermis	9
	Epidermal neoplasms of the mouse	9
	Induction	9
	Classification and histogenesis	11
	Mitotic activity and growth	14
	Microscopic anatomy of mouse lung	19
	Tracheo-bronchial tree	19
	Alveolar tissue.	20
	Pulmonary neoplasms of the mouse	23
	Alveologenic neoplasms	23
	Tracheo-bronchial neoplasms	26
	Measurement of mitotic activity	29
	Mitotic index	30
	Tritiated-thymidine index	31
	Colchicine technique	33

CHAPTER PAGE

III. MATERIALS AND METHODS 38
Test animals 38
Carcinogens used 38
Administration of the carcinogens 39
Experiment No. 1 39
Experiment No. 2 39
Experiment No. 3 39
Experiment No. 4 40
Experiment No. 5 40
Administration of colchicine 40
Sacrifice and histological technique 41
Experiment No. 1 41
Experiment No. 2 41
Experiment No. 3 42
Experiment No. 4 42
Experiment No. 5 43
Enumeration of nuclei 43
Statistical methods 46
IV. RESULTS 47
Experiment No. 1 47
Ear epidermis 47
Mitotic rates in untreated ears 47
Mitotic rates in treated ears 49
Interscapular epidermis 52

CHAPTER PAGE

Mitotic rates in untreated epidermis . . . 52

Mitotic rates in treated epidermis 52

Experiment No. 2 59

Interfollicular epidermis 59

Hair-follicular epithelium 63

Histogenesis 66

Mitotic rates 67

Experiment No. 3 75

Histology 78

Mitotic rates 80

Experiment No. 4 84

Histology 84

Experiment No. 5 85

Subcutaneous implants of methylcholanthrene 85

Subcutaneous implants of lung only 88

Lung tissue combined with methylcholanthrene 88

Mitotic rates 89

V. DISCUSSION 92

Experiment No. 1 92

Ear epidermis 92

Interscapular epidermis 95

Experiment No. 2 98

Interfollicular epidermis 98

Hair-follicular epithelium 99

CHAPTER PAGE

Experiment No. 3 103
Experiment No. 4 109
Experiment No. 5 111
VI. CONCLUSIONS 113
Experiment No. 1 113
Ear epidermis 113
Interscapular epidermis 113
Experiment No. 2 114
Interfollicular epidermis 114
Hair-follicular epithelium 115
Experiment No. 3 116
Experiment No. 4 116
Experiment No. 5 117
BIBLIOGRAPHY 118

CHAPTER I

INTRODUCTION

I. PROBLEMS

Experiment No. 1

Rates of Cell Division During the First 24-Hours of Benzpyrene Epidermal Carcinogenesis. I: Ear Epidermis, II: Interscapular Epidermis.

The purpose of this experiment was to determine the initial effect of a carcinogen on the mitotic activity of the epidermis.

Experiment No. 2

Rates of Cell Division During Benzpyrene Epidermal Carcinogenesis. I: Interfollicular Epidermis, II: Hair Follicular Epithelium.

The purpose of this experiment was to determine the pattern of mitotic activity during the development of an epidermal neoplasm.

Experiment No. 3

Mitotic Activity of the Alveologenic House Tumor.

The purpose of this experiment was (1) to estimate the daily rate of mitosis of the tumor, and (2) to estimate its doubling time.

Experiment No. 4

Failure to Induce Pulmonary Tumors by the Direct Application of Urethane to Pulmonary Tissue.

The purpose of this experiment was to induce pulmonary tumors by directly applying urethane to grafted mouse lung tissue.

Experiment No. 5

Rates of Cell Division of Pulmonary Tissue Grafts Containing Methylcholanthrene.

The purpose of this experiment was (1) to repeat Horning's (1947, 1950) technique of inducing bronchogenic carcinomas in mice, and (2) to estimate the mitotic activity of the induced neoplasms.

2. IMPORTANCE OF THE STUDY

The epidermis and the alveolar tissue of the lung are recognized examples of tissues that continually undergo a renewal of their cell populations. Cells are constantly dividing yet the cell number remains constant and unchanged. This cellular steady-state condition is an expression of a homeostatic or "growth-controlling" mechanism which ensures that the rate of cell production is balanced by an equal rate

cell loss (Bullough and Laurence, 1960; Bullough, 1962).

Certain carcinogens or their metabolic products react with these tissues to result in a cell population that progressively increases its cell number. The carcinogen apparently disturbs or deranges the "growth-controlling" mechanism. Poel, (1959) has suggested that during this homeostatic imbalance the cancerous cell makes its appearance.

Since mitotic activity of a tissue is an expression or reflection of this cyberenetic interaction between cells, it is believed that the disturbance of this mechanism can be followed by recording the rate of cell division prior to, during and after carcinogenic treatment.

Experiments No. 1, No. 2, and No. 3 are in a sense preliminary or exploratory experiments in this direction. They represent the beginning of a series of projects designed to investigate the influence of carcinogenic and non-carcinogenic agents on cellular steady-state systems.

The manner in which carcinogens convert normal cells into malignant ones, as alluded to previously is a central problem in present day cancer research. One aspect of this problem is ascertaining whether a carcinogen acts directly on the target cells or whether it undergoes some change in the body before reaching the tissue, so that its effect is through an intermediary substance or metabolite. In

Experiment No. 3, urethane, a carcinogen for mouse lung was injected intraperitoneally so as to accelerate the formation of alveologenic tumors. Its use by this route prompted the following question: would urethane still be capable of inducing lung tumors if it were directly applied to lung tissue? Experiment No. 4 represents an attempt to answer this question and may be considered a contribution to the mode of urethane's carcinogenic action.

With the rising incidence of human lung cancer it has become evident there there is a need for an experimental model of lung cancer in laboratory animals comparable to human bronchogenic cancer. Bronchogenic cancer in laboratory mammals can only be induced in sufficient number of animals with difficulty and following a considerable expense of time.

Experiment No. 5 was designed to emphasize a comparatively rapid and simple technique of induction. The technique is unorthodox in that it involves the removal of the lung from its natural environment. The acceptance of such a technique will depend largely on the morphological and behavioral resemblance of the resulting tumors with those that are induced by the more conventional methods. Such a project of comparison and evaluation would of necessity involve a considerable expense of time. At the present, only the rate of cell division of these tumors is reported.

In this thesis, considerable attention has been given

to the estimation of the rates of cell division of certain neoplastic tissues. The justification and continuance of such a field of study is perhaps emphasized by the following quotation:

> Extensive studies on the time parameters of cell proliferation, life span and death rates of cancer cells at various stages in the cancerous process in all types of neoplastic disease are urgently needed in parallel with construction of models of cell proliferation to explain the development of expanding tumor masses. Knowledge to be gained from these studies is essential for an understanding of the neoplastic process, its national therapy and prognosis. (Cronkite et al. 1962).

CHAPTER II

REVIEW OF THE LITERATURE

1. MICROSCOPIC ANATOMY OF MOUSE SKIN

(a) Epidermis. In Experiments No. 1 and No. 2 two structurally different regions of mouse epidermis were studied: interscapular and ear epidermis.

The interscapular epidermis is restricted to sites between adjacent hair follicles and is therefore appropriately referred to as interfollicular epidermis (Fig. 1). The structure and behavior of this region is considered to be important in that it may be the source of certain epidermal tumors. The cell population is three or four cell layers thick and consists of a layer of basal cells resting on the basement membrane, and differentiating cells above them. Cell division occurs in the basal cells, 50 per cent of the daughter cells are pushed upward to form a thin layer of keratin, while the other half are retained at the dermo-epidermal junction. Functionally, the interfollicular epidermis is a simple organ with two basic cell types: the basal cells in which proliferation occurs and the differentiating cells in which keratin formation takes place. The epidermis can therefore be considered as a holocrine keratin gland which produces cells that lose their ability to divide and are transformed into horny material.

Contrasted to the interfollicular epidermis, the epidermis of the ear pinna is thinner, consisting of one or

two cell layers covered over with a thin keratin lamella (Fig. 2). Moreover, there are relatively few hair follicles present. Functionally, the ear epidermis like the interscapular epidermis can be considered as a holocrine keratin gland.

Because of their comparative simplicity in structure and function, both the interscapular and ear epidermis represent ideal systems for studying cellular responses to carcinogens.

(b) Pilosebaceous apparatus. This term refers to the unit aggregation of a hair follicle, sebaceous gland and arrector muscle. The hair follicle and sebaceous gland are the source of certain skin tumors. The sebaceous adenoma was not studied in this report and therefore the structure of the sebaceous gland will not be examined. The hair follicle however, appears to be intimately related with the histogenesis, yield and growth of epidermal tumors of laboratory mammals (see Review, Argyris, 1963). The structure of the follicle is not constant but undergoes a cyclic variation which constitutes the hair follicular cycle. Each follicle has an independent cycle of downgrowth into the lower regions of the dermis where hair production occurs and then a stage of regression where the follicle retreats to the upper limits of the dermis. The downgrowth of the follicle and the production of hair is

Figure 1. Normal mouse interfollicular interscapular epidermis. The epidermis is three or four cell layers thick. Hair follicular cycle: Telogen Hematoxylin and eosin X 64

Figure 2. Normal mouse ear epidermis. Epidermis is one or two cell layers thick. Relatively few hair follicles are present. Hematoxylin and eosin X 260

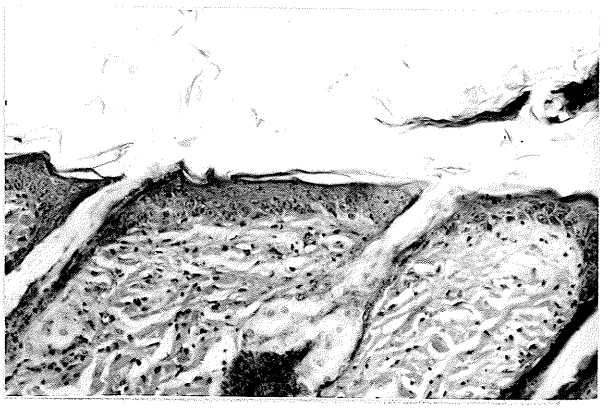

Figure 1.

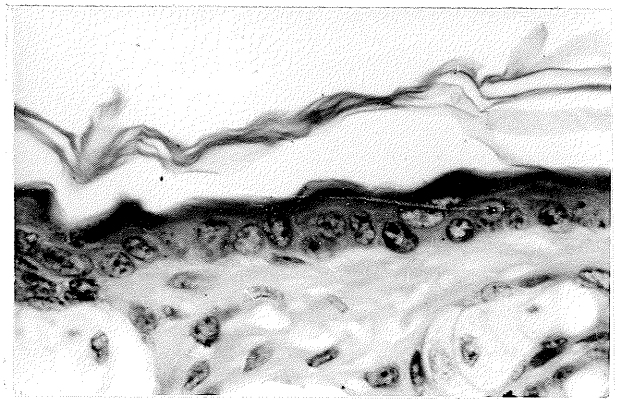

Figure 2.

referred to as the growth or anagen stage. This is followed by the cessation of hair formation and the degeneration of the lower half of the follicle (catagen). The dermal papilla moves upward and the resting or telogen stage of the cycle begins.

Argyris (1963) has reviewed the literature relating to the effect of hair growth cycles on skin tumorigenesis. He draws attention to the need for experimental studies relating to the evolution of skin tumors in relation to the hair cycle when multiple applications of a carcinogen are used.

(c) Dermis. While the dermis appears to be intimately involved in the histogenesis and establishment of epidermal tumors (Orr, 1963), this relationship was not studied in the present report.

The dermis consists largely of dense bundles of collagen fibres, irregularly arranged. No dermal papillae are present. Below the dermis is a thin stratum of adipose tissue which separates the dermis from the panniculus carnosus, a layer of striated muscle.

2. EPIDERMAL NEOPLASMS OF THE MOUSE

(a) Induction. It was 144 years after Percivall Pott's historic observation of scrotal cancer in chimney sweeps, that Yamagiva and Itchikawa in 1914 were able to

experimentally induce skin cancer in rabbits by repeated paintings with coal tar. A considerable advance was made in the late 1920's and early 1930's when the experiments of Kennaway, Hieger, Mayneord, Cook and Hewitt, (Kennaway, 1955) led to the identification of the carcinogenic substances in coal tar. With the synthesis of these polycyclic hydrocarbons, numerous experiments were designed to test their potency and examine their mode of action.

Three of the most widely used hydrocarbons employed to elicit skin cancer are 1,2-benzpyrene, 3-methylcholanthrene and 9, 10-dimethyl -1, 2-benzanthracene. Of these three, dimethylbenzanthracene is the most potent carcinogen for mouse, rabbit, rat and guinea-pig skin, (Berenblum, 1949). Since mouse skin is the most susceptible to the effect of carcinogens it has been used almost exclusively for the study of epidermal carcinogenesis.

The conventional method of inducing skin tumors has been the topical application of the carcinogen. The technique frequently used has been the painting of the skin with a small camel's-hair brush. Such a method has numerous disadvantages. Greater uniformity in administering the carcinogen can be achieved by the use of measured drop applications with a calibrated needle pipette. For a more precise control of the carcinogen reaching the skin, the area of the skin exposed and the time the carcinogen remains on the skin, the technique of

McCarter (1956) deserves to be considered.

To avoid undue manipulation of the skin and yet retain a quantitative and uniform method of induction other routes of administration have been employed. Ritchie (1957) showed that intraperitoneal injections of urethane would initiate epidermal cells. Recently, Poel (1963) has established that gastric intubation of methylcholanthrene and dimethyl-benzanthracene resulted in epidermoid carcinomas.

(b) Classification and histogenesis. The purpose of this discussion is to briefly review the most common types of experimentally induced epidermal neoplasms and their histogenesis. Sebaceous and melanotic tumors will not be discussed.

Benign epidermal tumors are referred to as papillomas while the term epidermal carcinoma is reserved for the malignant form. Chemically induced papillomas are more common than carcinomas, appearing earlier and usually regressing and disappearing during the lifetime of the animal. Because papillomas become visible shortly after treatment, the majority of studies on skin neoplasia have dealt with this form.

Papillomas have been classified into a number of sub types (Shubik et al. 1953; Ghadially, 1961). Ghadially's system is based on their histogenesis and will be described here.

He recognizes three types of papillomas, (1) Bud-shaped keratoacanthoma (Type 1), (2) Dome- or berry-shaped keratoacanthoma (Type 2) and (3) Primary papilloma.

Type 1 and Type 2 keratoacanthomas arise from the hair follicle: Type 1, from the region of the follicle above the sebaceous gland and Type 2 from the deeper part or hair germ of the follicle. The primary papilloma arises from the interfollicular epidermis and does not require hair follicles for its development. Ghadially, considers this type to be the classical benign tumor of glaborous epithelium. He believes that there is a remote possibility that this type could arise from rodent interfollicular epidermis, because the hair follicles are so closely packed that little interfollicular epidermis is exposed.

The most common type that arises in mouse skin is the bud-shaped or Type 1 keratoacanthoma. It arises as a result of the enlargement and fusion of the superficial parts of a group of hair follicles. Both Type 1 and Type 2 regress. The rate of regression of Type 1 is much slower and may take a number of paths. The most common one is an exfoliation of the tumor to produce a papillomatous structure which Ghadially calls a "secondary papilloma."

An interesting and plausible hypothesis has been put forward by Ghadially concerning the origin and behavior of the keratoacanthomas of rodent skin. He believes that Type 1 and

Type 2 are perverted products of the hair follicle but are still under the control of the forces that regulate the cyclic growth of the follicle. The keratoacanthomas do not extend any deeper in the dermis than the normal follicle. Moreover, the rapid growth and regression of the tumors parallels the cycle of hair growth.

Comparison of mitotic activity in the hair follicular epithelium and the keratoacanthomas during their cyclic growth may be of interest in testing this hypothesis. The study of the histogenesis of papillomas in hairless mice (Deringer, 1962) may provide further information concerning the role of the hair follicles in the genesis of epidermal tumors.

The most common type of carcinoma that arises in mouse skin is the squamous cell carcinoma. Glückomann (1945) considers that it may arise either from pre-existing papillomas or from hyperplastic epidermis. Using a viral infection in cortisone-treated mouse skins, Siegler and Duran-Reynals (1962) followed the development of methylcholanthrene induced papillomas and carcinomas. They found that carcinomas and papillomas did not co-exist at the same time. The squamous cell carcinomas did not arise directly from papillomas, but rather from the epidermal surface where the papillomas regressed. Ghadially (1961) drew attention to islands of epithelial cells that broke away from the base of Type 1 keratoacanthomas. These islands or columns of cells appeared

to mimic an early squamous carcinoma and he referred to them as "pseudocarcinomatous infiltrations." Occasionally, a true carcinoma may develop from them.

In summary: present evidence strongly suggests that experimentally induced papillomas in rodent skin require the presence of hair follicular epithelium. The induction of epidermal tumors in hairless mice does not contradict this conclusion, for as Argyris (1963) pointed out these mice have remnants of follicles, and the presence of skin tumours in these animals suggest that organized hair follicles may not be necessary for tumorigenesis.

Papillomas consist of a variety of morphological types. The diversity appears to be related to the stage of the hair cycle and the region of the follicle that becomes neoplastic (Ghadially, 1961). The histogenesis and development of carcinomas requires more study.

(c) Mitotic activity and growth. The literature relating to the measurement of mitotic activity of carcinogen treated epidermis will be reviewed under the following topics: (1) the effect of a single application of a carcinogen on the mitotic activity; (a) within the first 48-hours; (b) later than 48-hours; (2) the effect of repeated applications on mitotic activity, and (3) the mitotic activity and growth of induced epidermal tumors.

(1) The effect of a single application of a carcinogen on the mitotic activity (a) within the first 48 hours. Evensen (1961) observed that as soon as one hour after application of methylcholanthrene the number of mitotic figures had increased, being about three times greater than untreated epidermis. Twenty-four hours after application the mitotic index had decreased. Glücksmann (1945) reported an increase in mitotic figures from 6 to 24 hours after a single painting of benzpyrene. A single painting of methylcholanthrene to the ears of mice resulted in no significant change in the mitotic index during the first 12 hours (Heller and Cooper, 1944). From 12 hours onward Heller and Cooper found that the mitotic index rose above normal till 48 hours, when the experiment was terminated.

In the foregoing reports, consideration was not given to the effect of the sleep and awake cycle on the mitotic activity of the epidermis. The incidence of mitosis varies with this cycle (Cooper and Franklin, 1940; Bullough, 1948). Moreover, the use of the mitotic index, as a measure of mitotic activity may lead to an incorrect interpretation of the data (see "mitotic index"). In an attempt to control the sleep and awake cycle as well as obtain a more precise measure of a carcinogens initial effect on epidermal mitosis, Experment No. 1 was designed.

As to the effect of a single application of a

carcinogen, on mitotic activity later than 48 hours, a number of experiments are available. Pullinger (1940) reported a progressive increase in mitotic figures up to six days after the application of methylcholanthrene. Glücksmann (1945) found that a single painting of benzpyrene resulted in a steady increase in mitosis up to three weeks, at which time the incidence returned to normal levels. She also found that there was no inhibition of mitosis at any time. A similar stimulation of mitosis in mouse epidermis was observed by Daoust (1959). An inhibition of mitosis was not recorded during the 70 days of the experiment. The results of Dammert (1961) contradict the conclusion of a progressive stimulation of mitosis that follows a single painting. He observed an inhibition within the first week after a single treatment of dimethylbenzanthracene.

It is somewhat difficult to compare the results of these workers, since the use of different strains of mice, solvents and methods of administration complicate an analysis. Present evidence seems to indicate that a carcinogen progressively stimulates mitosis. The use of the mitotic index in these studies, may as previously mentioned, introduce a source of error.

(2) The effect of repeated applications of a carcinogen on the mitotic activity of the epidermis. Multiple

application has been used so as to accelerate the neoplastic process. The purpose of recording mitotic activity during the histogenesis of epidermal tumors is to determine if there is a characteristic pattern of mitosis. A distinct pattern appears to exist following the repeated application of coal tar to the skin of the mice (Champy and Vasiliu, 1923). Cooper and Heller (1942) administered a single application of methylcholanthrene to the ears of mice daily for 93 days. Ears were removed two days after the previous painting and whole mounts of the epidermis were prepared. The authors found, using the mitotic index, that the mitotic pattern during carcinogenesis consisted of progressive increase of mitosis, followed by a mid point depression and a terminal increase which accompanied the appearance of papillomas.

In an attempt to determine if a similar pattern existed when multiple applications of benzpyrene were applied, Experiment No. 2 was designed. Part I of the experiment emphasizes the extent of mitosis in interfollicular interscapular epidermis while in Part II, mitosis were recorded in the epithelial tissue of the hair follicles

(3) Mitotic activity and growth of induced epidermal neoplasms. Previous experiments have been designed (1) to estimate the growth rate of an epidermal tumor, (2) to determine whether the tumor has a higher or lower mitotic rate than untreated or hyperplastic epidermis, and (3) to

determine the presence or absence of a diurnal rhythm of cell division in the tumor.

Mottram (1934, 1936) observed that induced papillomas and carcinomas grew at the same rate. This finding could not be confirmed by Shubik et al. (1953), who observed that the growth rate of papillomas was not constant, but increased with time. Kiljunen (1956) using rat epidermal tumors found that carcinomas grew at a faster rate than papillomas.

Blumenfeld (1943) concluded that the rate of mitotic activity in mouse skin carcinomas was no greater than the maximum rate in an equal volume of normal epidermis. There was no evidence of a diurnal rhythm. Voutilainen (1953), however, found a day and night variation in mitosis in human skin cancer. In the skin of the hairless mouse, Evensen, and Iversen (1962) estimated the mitotic rate of a methylcholanthrene induced squamous cell carcinoma. The mitotic rate of the tumor was found to be less than that for untreated epidermis.

From the foregoing it appears that more information is required to determine if the growth rate of papillomas and carcinomas is constant throughout their lifetime. Evensen's and Iversen's (1962) unexpected observation requires confirmation in view of Blumenfeld's (1943) conclusions and recent observations made with other tumor systems (Bertalanffy 1963a and 1963b).

Experiment No. 2, Part II, represents an attempt to measure the mitotic activity of induced epidermal papillomas and carcinomas by means of the colchicine technique.

3. MICROSCOPIC ANATOMY OF MOUSE LUNG

(a) Tracheo-bronchial tree. The tracheo-bronchial tree is a collective term for the system of airways that conduct air from the larynx to the respiratory tissue and vice versa. These air passages are the trachea, the extrapulmonary bronchi, the intrapulmonary bronchi, bronchioles and alveolar ducts.

The following discussion briefly describes the epithelium that lines this system of air passages. The trachea is coated with pseudostratified ciliated columnar epithelium and it divides, forming two extrapulmonary bronchi which are structurally similar. The intrapulmonary bronchi, with their dichotomous branching lead to smaller bronchi lined with simple columnar ciliated epithelium. From these smaller bronchi arise the bronchioles. The first order are referred to as terminal bronchioles which bear non-ciliated cuboidal or low columnar cells. Goblet cells are absent. The terminal bronchioles give rise to the respiratory bronchioles which have a simple cuboid epithelium, devoid of cilia and goblet cells. At their termination, the respiratory bronchioles flare out giving rise to several alveolar ducts containing

groups of isolated cuboidal cells (bronchiolar cell rests). The alveolar ducts may for convenience be thought of as a long hallway from which rooms lead offfrom the sides, floor and ceiling (Ham and Lesson, 1961). These "rooms" are termed the alveoli. The partitions between adjacent alveoli are called the alveolar walls or inter-alveolar septa. Collectively they represent the respiratory tissue of the lung.

(b) Alveolar tissue. For many years the detailed architecture of the alveolar wall had been one of controversy (see Review, Bertalanffy, 1964). The present concept of this region of the lung has resulted from a series of electron microscope studies (Low, 1952, 1953; Karrer, 1956, 1958) and histochemical techniques. (Leblond and Bertalanffy, 1951, Bertalanffy and Leblond, 1953).

The following discussion is a brief review of the structure of the alveolar wall (Fig. 3). The cellular components of the alveolar tissue can for convenience, be classified into (1) those cells that are formed in the wall, the so-called inherent cells, and (2) leucocytes which by diapedesis enter the interstitial tissue of the septa.

In addition, to the capillary endothelial cell there are two distinct morphological and embryological cell types: (1) the alveolar cells, and (2) the pulmonary surface epithelial cell. Alveolar cells, often referred to in exfoliate cytology

Figure 3. Alveolar tissue of the lung. The basement membrane and the pulmonary surface epithelial cells have been enlarged in relation to the other elements of the alveolar tissue. The drawing was based on orth-fixed, Masson's trichrome stained sections. (From Ham and Leeson, Histology, 4th ed., 1961)

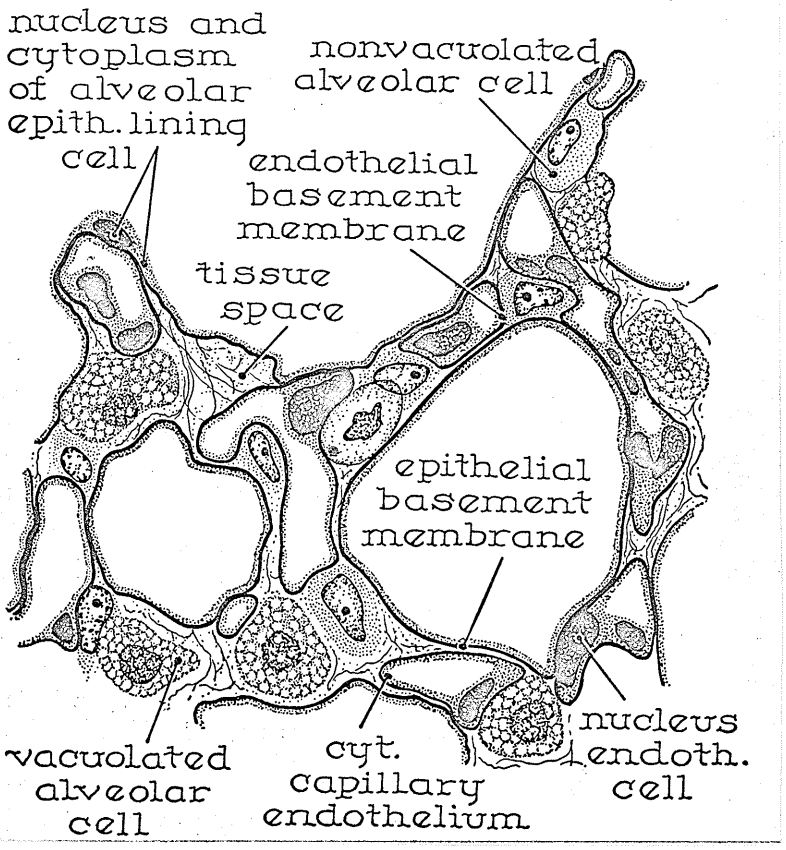
nucleus and cytoplasm of alveolar epith. lining cell
nonvacuolated alveolar cell
endothelial basement membrane
tissue space
epithelial basement membrane
vacuolated alveolar cell
cyt. capillary endothelium
nucleus endoth. cell

Figure 3.

as pulmonary macrophages, may be classified as (a) undifferentiated stem cells, (b) vacuolated alveolar cells or as (c) non-vacuolated alveolar cells. Both vacuolated and non-vacuolated types are capable of phagocytosing particulate matter. They thus represent the reticulo-endothelial system of the alveolar wall.

The alveolar cells are continually extruded from the wall, but the alveolar tissue, however, does not become depleted of these cells since new ones continually arise from pre-existing alveolar cells or by differentiation from the stem cell. Theoretically, one daughter cell is retained, the other is extruded so the alveolar tissue is maintained in a "steady state" with regard to its alveolar cell population (Bertalanffy and Leblond, 1953).

The pulmonary surface epithelial cells may be considered as the terminal extension of the epithelium of the tracheobronchial tree that comes to line the surface of the alveolar wall. In contrast to the mesenchymal origin of the alveolar cells, the surface pulmonary cells appear to arise from the lung bud entoderm.

Bertalanffy (1964) has recently published a comprehensive review of the literature relating to the cytology of the alveolar tissue.

4. PULMONARY NEOPLASMS OF THE MOUSE

The respiratory system of the mouse gives rise to two basic types of tumors. Those that arise from the alveolar wall and which are termed alveologenic tumors, (Mostofi and Larsen, 1951), and those which develop from the epithelium of the tracheo-bronchial tree and are appropriately referred to as tracheo-bronchial neoplasms.

In the mouse, the alveologenic neoplasm is more common as it appears spontaneously during the lifetime of certain strains, and it is more readily induced by chemical treatment than tracheo-bronchial tumors (Shimkin, 1955). The latter are not known to develop spontaneously; they are difficult to induce, and the induction period is longer (Shabad, 1962).

The alveologenic and tracheo-bronchial tumors constitute the experimental models used in the study of human lung cancer. Since both types were studied in this report a brief review of both tumors will be given. The alveologenic neoplasm has been studied for a number of years with a corresponding wealth of literature. Comprehensive reviews of this literature are available (Shimkin, 1955; Stewart, 1959).

(a) Alveologenic neoplasms. The alveologenic neoplasm is the most common pulmonary tumor that arises in mouse

lung either spontaneously or by experimental induction. There has been a great deal of controversy with regard to the histogenesis of these tumors. Many authors considered that the tumor may arise from the bronchial epithelium (Livingood, 1896; Jobling, 1910, Orr, 1947). Detailed serial sections (Grady and Stewart, 1940; Mostofi and Larsen, 1951) and electron microscope studies (Okada et al., 1962; Svoboda, 1962) have convincingly established the alveolar wall origin of the common mouse pulmonary tumor. From a morphological, rather than a histogenic basis, many authors refer to these tumors as pulmonary adenomas. Since this expression is common in the literature both terms are used here interchangeably. While the tumors arise from the alveolar wall it has not yet been established from which cell type the tumor takes origin. In the absence of this information it is not permissable to use the term "alveolar-cell tumors," in reference to the common pulmonary tumor of the mouse.

The pulmonary adenoma attracted a considerable amount of attention in the early period of cancer research because these tumors arose spontaneously during the lifetime of the mouse. The original reports were concerned with the incidence and frequency of appearance in inbred mice (Haaland, 1908; Jobling, 1910; Slye et al., 1914).

With the synthesis of pure chemical carcinogens interest in the tumor was revived. It soon became established

that those strains of mice which had a higher frequency of spontaneous pulmonary tumors had a correspondingly high incidence of induced tumors, (Cowen, 1947; Selbie and Thackray, 1948). Furthermore, there was no morphological or behavioral difference between spontaneous and induced pulmonary adenomas (Andervont, 1938, 1939a, 1939b). Nor was there a difference in morphology when different carcinogens or different routes of administration were used (Andervont and Shimkin, 1940).

The following discussion will briefly review the mitotic activity and growth of the mouse pulmonary adenomas. The earlier reports of the spontaneous tumor all noted the scarcity of mitotic figures, and it was thus concluded that the rate of growth was slow. The first quantitative evaluation of its growth was limited to the enumeration of external tumor nodules. The number of nodules were dependent on, (1) the strain of the mouse, (2) the carcinogen, and (3) the route of injection (Shimkin, 1955). The counting of nodules, as a measure of growth rate is not without certain limitations since several adjacent solitary nodules, each growing expansively, may become confluent. To overcome this disadvantage and yet measure the relative rate of growth, Telford and Jeffery (1953) have suggested the use of a planimeter to calculate total tumor volume.

The most extensive studies on the growth of the

alveologenic tumor have been published by Shimkin and Polissar (1955, 1958). They attempted to measure change in the total cell population of the alveolar wall following a single carcinogenic stimulus. They found that within a day following the injection there was an increase in the number of nucleated cells which reached a peak about four days when there was a 100 per cent increase. The authors did not establish whether this increase in cell number was a hyperplastic response, or what cells, if any, were dividing, or whether the cell increase was due to an invasion of macrophages. In an attempt to determine which cells of the alveolar wall give rise to the tumor it may be of value to repeat their experiment and record the mitotic response of the various cells.

Shimkin and Polissar (1958) followed the growth of the tumors and found that with an increase of time the growth rates decreased. Experiment No. 3 is an attempt to confirm this observation using a different technique as well as to ascertain if the mitotic rate of the alveologenic tumor is slower, faster or the same as the normal alveolar wall.

(b) Tracheo-bronchial neoplasms. Since the mitotic activity and growth of the alveologenic tumor was being studied (Experiment No. 3), it was considered to be of interest to compare their growth rate with that of those tumors arising from the epithelium of the tracheo-bronchial

tree. A search of the literature for techniques of inducing tracheo-bronchial tumors revealed that experiments concerned with their induction were motivated either from the need to assay various environmental agents for their neoplastic potentialities, or secondly from the need to establish an experimental model of lung cancer comparable to the human type.

The need for an experimental model of tracheo-bronchial cancer has been emphasized by the real increase in this form of human lung cancer and accordingly, numerous attempts have been made to successfully induce this neoplam in rodent lungs.

The most common method is the intratracheal instillation of the carcinogen (Niskannen, 1949; Della Porta et al. 1958; Pylev, 1961; Shabad, 1962; Herrold and Dunham, 1962). While this method is natural in that it involves the introduction of the carcinogen into the respiratory system by the normal route of contamination, the yield of tumors is low and the period of induction is lengthy (6 to 12 months).

Another widely used technique involves the insertion of carcinogenic agents directly into the lung tissue either by surgical implantation of the carcinogen (Blacklock, 1957; Blacklock and Burgan, 1962), or the direct insertion of carcinogen-soaked threads through the ventral body wall into the lungs (Andervont, 1937; Kuschner et al., 1956; Stevensen and von Haam, 1963; Brown, 1963). Unfortunately, while the

technique is comparatively simple to administer, the tumor yield is low and the length of induction is long.

Horning (1947, 1950) introduced a method of inducing tracheo-bronchial neoplasms in 10 to 12 weeks in a high percentage of the animals by impregnating pulmonary tissue in vitro with a carcinogen and transplanting the tissue to the subcutaneous tissue of homologous hosts.

Horning's method of inducing lung tumors is an extension of the technique developed by Smith, (see Review, Smith, 1949) as a means of testing chemical agents for carcinogenic properties. This technique of directly applying the carcinogenic agent to the tissue has been employed by Pan and Gardner (1948) who reported the induction of malignant tumors from grafts of mouse uterine cornua and cervices treated with methylcholanthrene. Using the same technique, Hughes (1949) succeeded in inducing transplantable carcinomas from fragments of adult bladder that was combined with methylcholanthrene.

While it is clear that the basic technique is satisfactory for inducing tumors in a short period of time and with a high frequency of success, this method however, of inducing tracheo-bronchial neoplasms appears to have been ignored in the literature for no experiments relating to the character or behavior of these tumors could be found.

It seemed desirable, then, to attempt to confirm

Horning's observations and to use his method as a means of securing a large number of tracheo-bronchial tumors in order to measure their growth rate. Experiment No. 5 is concerned with this problem.

5. MEASUREMENT OF MITOTIC ACTIVITY

The term mitotic activity refers to the extent of mitotic cell divisions in a cell population. Mitotic activity may be expressed as (1) the percentage of cell divisions present at any one time, or as (2) the percentage of divisions occurring during a given time interval. In the first case the measurement is expressed as an index, while in the second, the measurement is one of rate. The purpose of this discussion is to briefly outline two techniques of determining index and secondly, to describe in detail the colchicine technique which was the method used in this study to estimate the rate of cell division.

Before discussing the methods of measuring mitotic activity, the life history of a dividing cell is presented. The reproductive cycle of a cell (cell cycle) is defined as the average time interval between any one stage in the parent cell and the attainment of the corresponding stage in one or both daughter cells. This interval is also referred to as the generation time. A complete cell cycle consists of three stages of interphase and four of mitosis. After completing

telophase, the daughter nuclei enter the G_1 stage--the post-mitotic gap, where the synthesis of RNA and proteins but not of DNA, occurs. Following G_1, the cell enters a period of DNA synthesis, the S phase. After completing this phase, the cell stops making DNA and enters the premitotic gap, G_2, where the nucleus is prepared for its entrance into mitosis. The generation time as well as the duration of these stages, especially those of G_1, S and G_2 have been determined for a variety of cell populations (Hughes et al., 1958; Quastler and Sherman, 1959; Edwards et al., 1960; Mendelsohn et al., 1960; Sherman et al., 1961; Fry et al., 1961; Lipkin et al., 1962; Post et al., 1963).

The methods of measuring mitotic activity will now be discussed. First, the mitotic and thymidine index, which are used to estimate the percentage of cell divisions present at any one time.

(a) Mitotic index. The mitotic index or mitotic count is the percentage of mitotic figures present at any one instant of time. Many authors have used this term to refer to the number of mitotic figures per unit length of tissue. The two values are not necessarily similar and difficulty may arise in comparing the two. A serious disadvantage of the mitotic index is that its value is influenced by changes in the mitotic duration. A measured increase in the number of mitotic figures may not necessarily reflect an increase in mitotic activity but

rather a lengthening of the mitotic cycle.

(b) Tritiated-thymidine index. The doubling of DNA prior to mitosis involves the incorporation of DNA precursors into the macromolecule. Thymidine is one such precursor, and in the form of thymidylic acid it is taken up by nuclei synthesizing DNA (Reichard and Estborn, 1951). Furthermore, thymidine can be labelled with the isotope tritium which is a soft beta emitter, having a short range of one to three microns (Robertson et al., 1959). Injection of tritiated thymidine into an animal results in the label being incorporated during a time interval of approximately one hour that tritiated-thymidine is available (Friedkin et al., 1956; Cronkite, 1959). Unused thymidine that has not been incorporated, is quickly catabolized and eliminated from the body (Potter, 1959). Those cells that have become labelled are identified by radioautography.

The importance of identifying labelled interphase cells rests on the observation of Howard and Pelc (1951) that DNA synthesis occurs only in cells preparing for mitosis. By sacrificing the animal approximately one hour after injection, the percentage of labelled to non-labelled interphase cells can be ascertained. This figure is termed the tritiated thymidine or simply thymidine index. It may be defined as the percentage of cells synthesizing DNA at any one instant of time.

The index is a measure of mitotic activity since it gives an indication of the percentage of cells antipating mitosis.

The thymidine index does not suffer from the disadvantages of the mitotic index. Moreover, it is a much more sensitive measure of the percentage of dividing cells since present evidence indicates that the majority of cells involved in division at any one time are in interphase synthesizing DNA (Schultze and Oehlert, 1960). The mitotic index fails to recognize these cells. On the other hand, the thymidine index gives no indication of the percentage of mitosis present.

The tritiated thymidine index is based on what recently appeared to be a well established assumption, that is, a cell that has incorporated tritiated thymidine will eventually divide. Pelc (1963) has pointed out that there is a considerable amount of evidence for the uptake of thymidine in highly differentiated cells which is not associated with cell division. More recently, evidence has been presented that undifferentiated osteoblasts became labelled but did not divide. (Owen and MacPherson, 1963).

The rate of mitotic activity is expressed as the rate of mitotic cell division or more simply as the rate of mitosis. The rate of mitosis is the percentage of cells that enter mitosis during a given time interval. There are two techniques of estimating mitotic rate: an indirect method using tritiated thymidine and a direct one employing colchicine. The tritiated

thymidine method is an extension of the thymidine index where the percentage of cells synthesizing DNA per unit time is measured. The rate of DNA synthesis gives an estimate of the rate at which interphase cells enter mitosis. The colchicine method, the one used in the present study, will be discussed in detail in the following paragraphs.

(c) Colchicine technique. Colchicine is an alkaloid extracted from the plant Colchicum autumnale. It has the characteristic property of arresting mitotic cell division of metaphase by inhibiting the formation of the spindle (Eigsti and Dustin, 1955). This property has resulted in the drug becoming a valuable tool for the study of mitotic activity in plant and animal tissues (Eigsti and Dustin, 1955; Leblond and Walker, 1956; Bertalanffy and Lau, 1962).

During its presence in the tissue, colchicine causes a progressive accumulation of metaphase figures ("colchicine metaphases") by inhibiting cells that enter metaphase. Determination of the percentage of such metaphase nuclei that have accumulated between the time of injection and sacrifice permits an estimation of the mitotic rate (Leblond and Walker, 1956). Knowing the time required for a given percentage of cells to divide permits an estimate of the time need for 100 per cent of the cells to divide. In those cell populations characterized by cell renewal (Leblond and Walker, 1956; Bertalanffy and Lau, 1962), this result is expressed

as the renewal or turnover time. In contrast, those populations which grow expansively and where few cells are lost, as for example some tumors, the time interval in which all cells of the population divide is referred to as the doubling time (Kisieleski et al., 1961). A criticism of colchicine doubling times is presented in the discussion of Experiment No. 3.

The colchicine technique is not difficult to administer and any variations in mitotic duration that may occur are taken into consideration.

What follows is a discussion dealing with some possible sources of error in the interpretation of colchicine mitotic rates.

(1) Does colchicine accelerate or depress a cell's entrance into mitosis? This possibility has been tested by enumerating the percentage of prophases in ilial epithelium of colchicine treated and untreated rats. (Stevens Hooper, 1961). Noting that prophase incidence to be identical in colchicine treated and untreated animals Stevens Hooper (1961) concluded that "mitosis is neither stimulated nor inhibited since the presence of colchicine did not change the normal rate of initiating prophases, nor its duration."

(2) Does colchicine arrest all cells that enter metaphase? Colchicine's effect depends on the dosage of the drug and the time it remains in the tissue. Should anaphases

and telophases occur, cells are escaping from colchicine's action. Such escape may indicate that the dosage is too small or the period of action is too short. On the other hand, an excessively large dosage will elicit cellular damage. If the period between colchicine administration and sacrifice of the animals is excessively long (e.g. 9 to 12 hours and longer) many of the colchicine metaphases disintegrate and become lost from the count.

A dosage of 0.10 mg. per 100 gm. body weight was found to be optimal for estimating mitotic rates of rat tissues (Leblond and Walker, 1956). Mice require twice this dosage of colchicine to ensure the arrest of all mitosis (Bertalanffy and Leblond, 1953). Pilot experiments in this laboratory indicated that 0.20 mg. per 100 gm. body weight was optimal for arresting mitosis in neoplastic cell populations of the mouse.

Selection of a suitable time interval of colchicine action is determined in part by the type of tissue. For the gastric and ilial epithelia of the rat, a period greater than four hours introduces error due to disintegration and loss of colchicine metaphases (Stevens Hooper, 1961). Bertalanffy and Lau (1962) stated that a time period of 4 to 6 hours of colchicine action is sufficient for the estimation of mitotic rates of the majority of mouse and rat tissues. An interval of four hours may be more suitable than one of six for the estimation of mitotic rates in tissues with a

high mitotic incidence, since the probability of arrested metaphases disintegrating and being lost from the count is decreased.

(3) Is colchicine's action instantaneous? The presence or absence of a lag period appears to depend in part on the type of tissue and the route of administration. For ilial epithelium there is a lag period of 15 to 30 minutes following subcutaneous injection. Intravenous injection however, erases this lag (Stevens Hooper, 1961). In rat plantar epidermis there is a 30 minute lag period (Storey and Leblond, 1951). An appreciable lag period may introduce a considerable error into the interpretation of the results, especially those obtained from four consecutive six-hour intervals.

(4) Is colchicine acting evenly over the experimental period? The possibility exists that colchicine may not be arresting mitosis uniformly during the presence in the tissue. After administration of the full dose in a single injection, the concentration of colchicine may reach a peak at which its full effect became apparent, while before or after the peak, the effect would be less. A significant difference was not observed between the mitotic rate obtained after administration of six-hourly injections of one-sixth of a single dose and that obtained following an injection of a

single total dose (Bertalanffy and Leblond, 1953; Stevens Hooper, 1961).

(5) It has been maintained that if cells had not been arrested in metaphase, they would pass into interphase and re-divide during the six hour period of the experiment. Thus, colchicine rates would not be a reliable estimate of the percentage of dividing cells in a six hour period. For a cell to re-divide during a six hour period would require a generation time less than or equal to six hours. Such a remarkably short replication time has yet to be reported (see Table III, Post et al., 1963). Moreover, evidence has been put forward that the S phase for many somatic mammalian cells has a constant range of five to eight hours (Cameron and Greulich, 1963; Cameron, 1964).

In summary, the colchicine technique when administered with suitable controls as to dosage, duration of action, and lag period provides a reliable method for the quantitative estimation of mitotic rate. The results, however, represent a minimum value since (1) prophases are included with the interphase count and, (2) there may be a loss of some colchicine metaphases due to their fragmentation.

CHAPTER III

MATERIALS AND METHODS

1. TEST ANIMALS

Two strains of mice were used: (1) Male, Swiss strain, six to eight weeks of age and weighing an average of 25 gm. at the beginning of the experiments, (Manor Farms, Staatsburg, New York), (2) Male and female, strain A mice, which at the beginning of the experiments were six to eight weeks of age and weighed an average of 20 gm, (Roscoe B. Jackson Laboratory, Bar Harbor, Maine).

The animals which received Victor Fox food pellets and tap water ad libitum, were kept in metal cages, not more than four mice in each. During the experiments the temperature range was 80 to 85°F. The animals were exposed to the normal daily cycles of light and darkness.

2. CARCINOGENS USED

Three chemical compounds, all recognized for their ability to produce benign and malignant neoplasms in a variety of tissues were used (Hartwell, 1951; Shubik and Hartwell, 1957). The three compounds were: 1, 2-benzpyrene, (L. Light and Co., Colnbrook, England); 2, ethyl carbamate (urethane), (Fisher Scientific Co., Fairlawn, New Jersey); 3, methylcholanthrene, (Eastman Organic Chemicals, Rochester 3, New York).

3. ADMINISTRATION OF THE CARCINOGENS

(a) Experiment No. 1. In parts I and II, the epidermis of the experimental animals received a single painting with a No. 4 camel's hair brush of a 0.3 per cent solution of benzpyrene in acetone. The painting was done at 10 a.m. and the brush was dipped once into the solution and immediately applied. In part I, the medial surface of the right ear pinna of 12 strain A male mice was painted, while in part II the brush was applied to the unshaven epidermis at the nape of the neck and drawn caudally to the interscapular region. Sixteen male Swiss mice were treated.

Twelve untreated strain A and 16 Swiss mice were maintained as controls in I and II, respectively.

(b) Experiment No. 2. In part I, the unshaven interscapular epidermis of male Swiss mice was painted with a single stroke of No. 4 camel's hair brush containing a 0.3 per cent solution of benzpyrene in acetone. The paintings were administered at 10 a.m., twice a week (Monday and Friday) for 11 consecutive weeks. Three untreated Swiss mice served as controls. In part II, a similar technique of application was used except multiple strokes (five or six) were applied each Monday and Friday. Treatment continued for 25 consecutive weeks.

(c) Experiment No. 3. Sixteen male A strain mice were

given a single intraperitoneal injection of a 10 per cent aqueous solution of urethane (1.0 mg. per 100 gm. body weight).

(d) Experiment No. 4. Female strain A mice were sacrificed, and small pieces of lung were isolated and in vitro wrapped about a few crystals of urethane (approximately 1.3 mg.) and then inserted into the subcutaneous tissue of anaesthetised female strain A hosts. Sixteen hosts were used, each mouse received one or two implants in the ventral abdominal wall. A total of 28 grafts were inserted.

(e) Experiment No. 5. The method of administering the carcinogen was similar to that of Experiment No. 4. The only difference was that lung tissue was impregnated with methylcholanthrene crystals (approximately 0.2 mg.). Thirty mice received one or two implants. To serve as controls, five mice received a single implant of lung tissue without the carcinogen and to seven other animals a few crystals of methylcholanthrene (approximately 0.2 mg) were deposited directly into the superficial fascia.

4. ADMINISTRATION OF COLCHICINE

Each mouse of the experimental and control groups received, six hours prior to sacrifice, a subcutaneous injection of a 0.1 per cent aqueous solution of colchicine (0.2 mg. per 100 gm. body weight). The colchicine was

obtained from Inland Alkaloid Inc., Tipton, Indiana. The injection site was immediately caudal to the interscapular region.

5. SACRIFICE AND HISTOLOGICAL TECHNIQUE

All mice were sacrificed by chloroform anaesthesia six hours after colchicine administration. In Experiments No. 1 and No. 2, the tissues were fixed in Davidson's fixative. Sanfelice's fixative was used in Experiments No. 3, No. 4, and No. 5. All tissues were routinely prepared and paraffin sections were cut at 7μ and stained with hematoxylin and eosin. Mitotic counts were taken from every tenth section.

(a) Experiment No. 1. In parts I and II, the first group of control and experimental animals were sacrificed at 4 p.m. and the second group at 10 p.m., the same day. The third and fourth groups were sacrificed at 4 a.m. and at 10 a.m. respectively, the following day. Each group was taken to represent the percentage of mitosis occurring for a given six-hour period. Summation of the four groups permitted an estimate of the percentage of cells dividing during one 24-hour period, that is, the daily rate of mitosis.

(b) Experiment No. 2. On the seventh day following

the beginning of treatment and every seventh day thereafter mice were withdrawn from treatment and sacrified the next day. By following such a procedure the interval between paintings was kept constant as well as the interval between the last application of the carcinogen and the removal of the tissue. To maintain a constant period of observation all animals were sacrificed at 4 p.m. Thus, the estimate of the percentage of mitosis occurring at weekly intervals during the carcinogenic treatment was based on a constant six-hour period--10 a.m. to 4 p.m.

(c) Experiment No. 3. The 16 mice which had received a single intraperitoneal injection of urethane were sacrificed 209 days later. To determine the daily rate of mitosis and the presence or absence of day and night variations in mitotic activity, the 16 animals were divided into four groups of four animals. Each group represented a six-hour period of the 24-hour day. The first group was sacrificed at 4 p.m., the second at 10 p.m. the same day, while the third and fourth groups were sacrificed at 4 a.m. and 10 a.m. respectively, the following day.

(d) Experiment No. 4. The 16 mice bearing lung grafts impregnated with urethane crystals were sacrificed at varying intervals after implantation, namely, after 77 days, two

mice, after 105 days, two mice, after 212 days, five mice and after 343 days, seven mice.

(e) Experiment No. 5. Of the 30 mice implanted with methylcholanthrene contaminated lung grafts, five mice died during the experiment and were excluded from study. Twenty-five of the animals were sacrificed 109 days from the beginning of treatment during a 24 hour period. The mice were divided into four groups, each group representing a six-hour period. They were injected with colchicine and sacrificed according to the same scheme as outlined in Experiments No. 1 and No. 3.

Of the five mice which received lung graft only, one animal was sacrificed 115 days after treatment, while of the remaining four, two were sacrificed at 211 days and two after 344 days following implantation.

Of the seven mice which methylcholanthrene crystals were deposited into the subcutaneous tissue, two were sacrificed 105 days later, while the remaining five were allowed to live for 211 days.

6. ENUMERATION OF NUCLEI

All sections, except the lungs of Experiment No. 4 were examined with a binocular light microscope at a magnification of 650X. The lungs were studied with an oil

immersion objective. Using two hand tally counters more than 2000 distinct nuclei were counted. They were numerically classified as colchicine metaphases or interphase nuclei. Prophases were included with the interphase count. Anaphases and telophases were not observed. Colchicine metaphases were recognized by their, (1) darkly staining clumps of chromatin, (2) peripheral halo of cytoplasm, (3) swollen cell outline, and (4) absence of the nuclear membrane, (Fig. 4). Not all colchicine metaphases, however, had these features combined. In many nuclei the individual chromasomes could be seen. Some difficulty was experienced in distinguishing nuclei with pyknosis and karyorrhexis from colchicine metaphases. In the majority of cases the swollen cell outline and the peripheral halo of cytoplasm about the nuclear material were indicative of colchicine blocked cells.

In Experiment No. 1 more than 4000 interfollicular epidermal nuclei were counted per animal. Enumeration began at one end of the section and continued until all representative areas of the section had been included in the count.

In Experiment No. 2, part I, all distinct interfollicular epidermal nuclei were counted while in part II the epithelial cells of the hair follicles were counted. In hyperplastic states, all distinct nuclei from the basal layer up were included in the count. The papillomas and carcinomas

Figure 4. Three colchicine metaphases howing the characteristic features of (1) a central dark staining chromatin clump, (2) halo around the chromatin, (3) swollen cell outline, and (4) absence of a nuclear membrane. The tissue is from an epidermoid carcinoma of the skin. Hematoxylin and eosin X 500

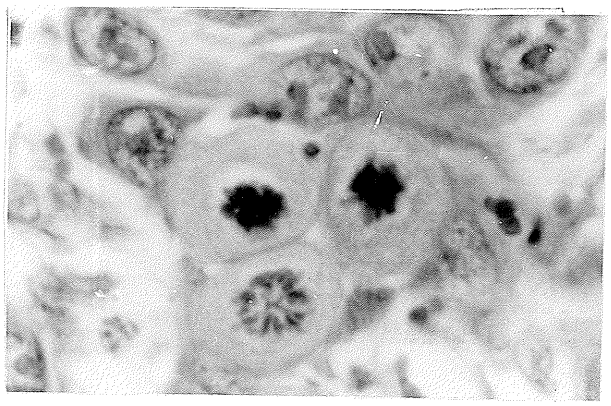

Figure 4.

were traversed from one edge to another so as to include cells in the peripheral as well as central areas.

Counts were not recorded in Experiment No. 4. The counting of nuclei in the induced tumors of Experiment No. 5 was so arranged to include representative areas.

7. STATISTICAL METHODS

For the calculation of the arithmetic mean and the standard deviation of a series of mitotic counts, the two following formulae were used: $\bar{x} = \sum x_1/n$ and $s = \sqrt{\sum x_1^2/n - \bar{x}^2} \times \sqrt{n/n - 1.}$ The stastic $\sqrt{n/n - 1}$, is a correction factor for small samples (Hill, 1955).

Two tests of significance were used: the "t" test

$$\left\{ t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2}{n_1 + n_2 - 2} \left(\frac{1}{n_1} + \frac{1}{n_2} \right)}} \right\}$$

and the analysis of variance. For the latter, the formulae and method were taken from Croxton (1958).

A 5 per cent level of significance was used to accept or reject the null hypothesis.

CHAPTER IV

RESULTS

1. EXPERIMENT NO. 1

(a) I: Ear epidermis. The purpose of this experiment was to determine the effect of a single painting of benzpyrene on the mitotic activity of the epidermis of the mouse ear pinna during the first 24 hours of benzpyrene carcinogenesis.

A single painting of the carcinogen was administered to the ears of 12 mice at 10 a.m. The mitotic rates of the following four consecutive six-hour intervals were estimated by the colchicine technique. To determine the mitotic rates of the corresponding intervals in untreated ears, 12 mice were not painted with the carcinogen. They were injected with colchicine and sacrificed at identical times as the treated animals.

(i) Mitotic rates in untreated ears. The mitotic rates of the four, six-hour periods during a 24-hour period is shown in Table I. The table indicates that the percentage of cells entering mitosis is not constant, but varies with the time of day. Fewer mitosis occurred during the interval from late afternoon to early morning (4 p.m. to 4 a.m.). Using the mitotic index, Cooper and Franklin (1940) previously reported a diurnal rhythm for mouse ear epidermis. The present results confirm their observations.

The metaphase nuclei appeared to be evenly distributed over the entire ear, for there was no evidence of localized

TABLE I

MITOTIC RATES OF UNTREATED EAR EPIDERMIS DURING A 24-HOUR PERIOD

Time of Day	Nuclei No.	Metaphase No.	Per cent of Metaphases	
10 A.M.-4 P.M.	4038	44	1.09	1.56 ± 0.42
	4110	80	1.95	
	4026	66	1.64	
4 P.M.-10 P.M.	4060	28	0.69	0.74 ± 0.04
	4084	32	0.78	
	4020	30	0.75	
10 P.M.-4 A.M.	4008	28	0.70	0.81 ± 0.07
	4028	32	0.79	
	4098	38	0.93	
4 A.M.-10 A.M.	4026	68	1.69	1.64 ± 0.37
	4038	52	1.29	
	4018	78	1.94	

Daily mitotic rate = 4.75 ± 0.90

areas of cell proliferation.

Summation of the four means yields 4.75 per cent, which is an estimate of the percentage of cells that enter mitosis during the 24-hour period.

(11) Mitotic rates in treated ears. The mitotic rates of the four, six-hour intervals following the single application of benzpyrene at 10 a.m. is indicated in Table II. The daily mitotic rate was 3.09 per cent which indicates that 34 per cent fewer epidermal cells divided in a 24-hour period following the carcinogenic treatment than in untreated ears. This percentage decrease is statistically, highly significant ($P < 0.001$).

The pattern of mitotic activity in treated and untreated ears is shown in Fig. 5. During the first two six-hour periods the average mitotic rate in the experimental animals was slightly less, but this difference was not statistically significant. For the last two six-hour periods the average mitotic rate was depressed below the level of the controls. This decline in mitosis was statistically significant ($0.02 < P < 0.05$ and $0.001 < P < 0.01$ respectively). It was during the last six-hour interval, 18 to 24 hours after painting that the lowest average mitotic rate was recorded. This figure was approximately half of the lowest value found in the controls.

Foci of cell proliferation were not observed.

TABLE II

MITOTIC RATES OF EAR EPIDERMIS DURING THE FIRST 24-HOURS OF BENZPYRENE CARCINOGENESIS

Time of Day	Nuclei No.	Metaphase No.	Per cent of Metaphases	
10 A.M.-4 P.M.	4018	60	1.49	1.49 ± 0.27
	4036	72	1.78	
	4022	48	1.19	
4 P.M.-10 P.M.	4030	30	0.74	0.66 ± 0.35
	4208	28	0.66	
	4064	24	0.59	
10 P.M.-4 A.M.	4026	24	0.60	0.59 ± 0.09
	4068	25	0.61	
	4040	23	0.57	
4 A.M.-10 A.M.	4066	18	0.44	0.35 ± 0.08
	4020	14	0.35	
	4014	10	0.25	

Daily mitotic rate = 3.09 ± 0.79

Figure 5. Mitotic pattern in benzpyrene-treated and untreated mouse ear epidermis during a 24-hour period. Each bar represents the percentage of epidermal cells entering mitosis in a six-hour interval.

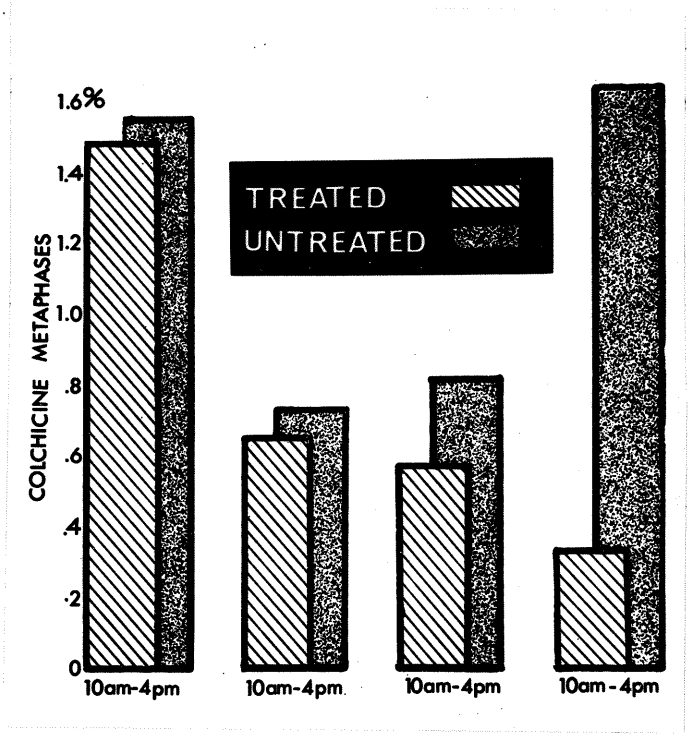
1.6%
1.4
1.2
1.0
.8
.6
.4
.2
0
COLCHICINE METAPHASES
TREATED
UNTREATED
10am-4pm
10am-4pm
10am-4pm
10am-4pm

Figure 5.

(b) II: Interscapular epidermis. The purpose of this experiment was similar to that of part I. The interscapular epidermis was selected because (1) it is the region most often used to induce epidermal tumors and, (2) its structure differs significantly from that of ear epidermis by the presence of abundant hair follicles.

(i) Mitotic rates in untreated epidermis. The mitotic rates of the four, six-hour periods is indicated in Table III. One animal died during the interval from 4 a.m. to 10 a.m. and was excluded from the experiment.

The table indicates that there was a considerable variation of mitotic rates among the animals within each group. Analysis of variance revealed that there was no statistically significant difference among the four means.

In addition to a higher daily mitotic rate, interscapular epidermis had a greater degree of variation in mitotic activity than ear epidermis. The daily mitotic rate in interscapular epidermis was approximately 17 per cent (± 9.56) compared to 4.75 (± 0.90) in the ear.

In many of the animals, there were concentrations of colchicine metaphases in and around the mouths of the hair follicles.

(ii) Mitotic rates in treated epidermis. The daily rate of mitosis was 13.99 (± 7.94). A statistically

TABLE III

MITOTIC RATES OF UNTREATED INTERSCAPULAR EPIDERMIS DURING A 24-HOUR PERIOD

Time of Day	Nuclei No.	Metaphase No.	Per cent of Metaphases	
10 A.M.-4 P.M.	4178	156	3.73	3.79 ± 2.79
	4688	371	7.91	
	4067	92	2.26	
	4177	54	1.29	
4 P.M.-10 P.M.	4145	121	2.91	2.30 ± 0.85
	4336	57	1.31	
	4140	128	3.09	
	4080	78	1.91	
10 P.M.-4 A.M.	4267	311	7.28	4.25 ± 2.26
	4040	172	4.25	
	4074	78	1.91	
	4061	147	3.61	
4 A.M.-10 A.M.	4107	339	8.25	6.74 ± 3.66
	* -	-	-	
	4135	303	7.32	
	4147	193	4.65	

* died

Daily mitotic rate = 17.08 ± 9.56

significant difference did not exist between the daily mitotic rates of treated and untreated interscapular epidermis ($0.4 < P < 0.5$). The pattern of mitotic activity in both groups was similar (Fig. 6).

An analysis of variance revealed that there was no statistically significant difference among the four means of the experimental animals. The only statistically significant difference between the average mitotic rates of the control and experimental groups was in the 4 p.m. to 10 p.m. interval ($0.02 < P < 0.05$).

The individual mitotic rates of the experimental animals is recorded in Table IV.

Because of the unexpected variation in mitotic rates in both control and experimental animals a search for a possible factor or factors was undertaken.

The foci of colchicine metaphases in the follicular mouth as well as the various stages of the hair cycle that the skin sections presented, suggested that the growth of the hairs may have influenced mitotic activity in the interfollicular epidermis of both treated and untreated animals. Accordingly, the mitotic rates were compared to the stages of the hair cycle (Tables V, VI). The tables indicate that higher mitotic rates were found in areas of the skin in which the hair follicles were in the growth stage and lower rates were recorded in skin with resting follicles. The

Figure 6. Mitotic pattern in benzpyrene treated and untreated mouse interfollicular interscapular epidermis during a 24-hour period. Each bar represents the percentage of epidermal cells entering mitosis in a six-hour interval.

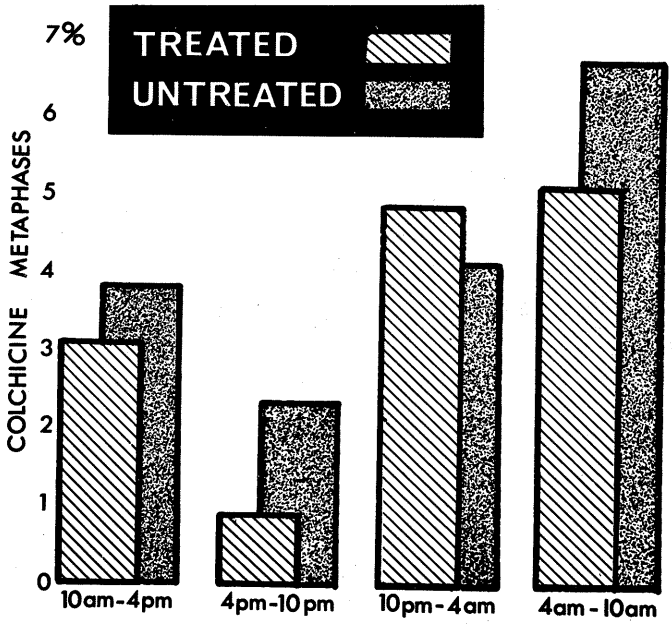
7%
6
5
4
3
2
1
0
COLCHICINE METAPHASES
TREATED
UNTREATED
10am-4pm
4pm-10pm
10pm-4am
4am-10am

Figure 6.

TABLE IV

MITOTIC RATES OF INTERSCAPULAR EPIDERMIS DURING THE FIRST 24-HOURS OF BENZPYRENE CARCINOGENESIS

Time of Day	Nuclei No.	Metaphase No.	Per cent of Metaphases	
10 A.M.-4 P.M.	4049	122	3.01	3.04 ± 2.09
	4123	224	5.43	
	4341	13	0.29	
	4224	144	3.41	
4 P.M.-10 P.M.	4092	59	1.44	0.98 ± 0.30
	4071	29	0.71	
	4078	39	0.96	
	4071	32	0.79	
10 P.M.-4 A.M.	4033	149	3.69	4.79 ± 1.46
	4074	246	6.04	
	4047	246	6.08	
	4075	136	3.34	
4 A.M.-10 A.M.	4038	65	1.61	5.18 ± 4.09
	4062	77	1.90	
	4039	404	10.00	
	4405	317	7.20	

Daily mitotic rate = 13.99 ± 7.94

TABLE V

RATES OF CELL DIVISION IN UNTREATED INTERSCAPULAR EPIDERMIS AND THE CORRESPONDING STAGES OF THE HAIR CYCLE

Time of Day	Per cent Metaphases	Stages of Hair Cycle
10 A.M.-4 P.M.	3.73	Anagen VI, I
	7.91	Anagen II
	2.26	Anagen V, VI
	1.29	Telogen
4 P.M.-10 P.M.	2.91	Anagen I
	1.31	Telogen
	3.09	Anagen I
	1.91	Telogen
10 P.M.-4 A.M.	7.28	Anagen II
	4.25	Anagen I
	1.91	Anagen VI
	3.61	Anagen IV
4 A.M.-10 A.M.	8.25	Anagen II
	*	-
	7.32	Anagen II
	4.65	Anagen I

* died

TABLE VI

RATES OF CELL DIVISION IN TREATED INTERSCAPULAR EPIDERMIS AND THE CORRESPONDING STAGES OF THE HAIR CYCLE

Time of Day	Per cent Metaphases	Stages of Hair Cycle
10 A.M.-4 P.M.	3.01	Telogen
	5.43	Anagen II
	0.29	Telogen
	3.41	Anagen II
4 P.M.-10 P.M.	1.44	Anagen II
	0.71	Anagen I, II
	0.96	Anagen I
	0.79	Anagen V, VI
10 P.M.-4 A.M.	3.69	Anagen II
	6.04	Anagen I
	6.08	Anagen V, VI
	3.34	Anagen II
4 A.M.-10 A.M.	1.61	Anagen I, VI
	1.90	Anagen II
	10.00	Anagen II
	7.20	Telogen

significance of this observation and its influence on the interpretation of mitotic rates will be examined in the discussion.

2. EXPERIMENT NO. 2

(a) I: Interfollicular epidermis. The purpose of this experiment was (1) to record the mitotic rate of interfollicular interscapular epidermis at weekly intervals during the development of an epidermal tumor.

Twice a week, for a period of 11 consecutive weeks, a single painting of benzpyrene was administered to the unshaven interscapular epidermis. Mitotic activity was estimated by the colchicine technique.

By the eleventh week, interscapular epidermis had been removed from a total of 32 treated animals, three being studied each week, with the exception of the eighth, when only two animals were examined. The third animal died immediately following the colchicine injection and was therefore excluded from the experiment.

The average mitotic rate for the six-hour period (10 a.m. to 4 p.m.) in the three control mice was 1.78 ($\pm$ 0.23) which was less than that observed in Experiment No. 1. The variation is attributed to the growth of the hair follicles.

The weekly mitotic rates during the 78 days of the experiment is shown in Table VII.

TABLE VII

MITOTIC RATES DURING BENZPYRENE EPIDERMAL CARCINOGENESIS
I: INTERFOLLICULAR EPIDERMIS

Mouse No.	No. of Paintings	Nuclei No.	Metaphase No.	Per cent Metaphase	
1		4044	42	1.03	
2	2	4062	119	2.93	2.31 ± 1.12
3		4028	120	2.98	
4		4056	99	2.44	
5	4	4199	12	0.28	1.29 ± 1.22
6		4318	50	1.16	
7		4064	35	0.86	
8	6	4167	29	.70	1.15 ± 0.63
9		4026	76	1.89	
10		4138	142	3.43	
11	8	4044	105	2.60	3.90 ± 1.58
12		4110	233	5.67	
13		4230	321	7.59	
14	10	4221	169	4.00	6.01 ± 1.89
15		4093	264	6.45	
16		4069	121	2.97	
17	12	4138	343	8.28	4.94 ± 2.89
18		4140	148	3.57	
19		4217	308	7.30	
20	14	4775	390	8.17	7.88 ± 1.46
21		4199	343	8.17	
22		4082	113	2.77	
23	16	4407	139	3.15	2.96 ± 0.28
24*		-	-	-	
25		4226	169	4.00	
26	18	4135	139	3.29	4.13 ± 0.90
27		4271	218	5.10	

* died

TABLE VII (continued)

Mouse No.	No. of Paintings	Nuclei No.	Metaphase No.	Per cent Metaphase	
28		4218	134	3.18	
29	20	4251	370	8.70	4.86 ± 2.32
30		4241	115	2.71	
31		4054	279	6.88	
32	22	4124	243	5.89	6.38 ± 0.77
33**		4226	429	10.00	

** papilloma (keratoacanthoma, type 2)

At the eighth day following two benzpyrene paintings the mitotic rate was similar to the controls. From the eighth to the twenty-second day, the average mitotic rate declined and by the twenty-second day, after six paintings, the average rate was lower than the controls. This depression was not statistically significant.

From the twenty-second day onward, the mitotic rate steadily increased, except for a slight depression at the forty-third day, to an average value of 7.88 per cent by 50 days. One week later, after two additional paintings, the average mitotic rate rapidly fell to a value of 2.96, which is a percentage decrease from the previous week of approximately -62 per cent.

The mitotic rate progressively recovered for the next three weeks when the experiment was terminated. The last animal group had received a total of 22 single benzpyrene paintings. In one of the three animals of the last group, a papilloma was found (Fig. 7) and classified on the basis of Ghadially's (1958; 1959; 1961) description as a keratoacanthoma, Type 2. It had a mitotic rate of 10 per cent.

The mitotic pattern in interfollicular interscapular epidermis during repeated paintings of benzpyrene appears to consist of four stages (Fig. 8). There is (1) an initial lag period, (2) a period of progressive cell increase, (3) a period of depression followed by (4) a terminal period of increased mitosis.

Figure 7. Keratoacanthoma Type 2, subtype B. The crater like lesion extends down to the panniculus carnosus. The central region of the tumor consists of branching epithelial columns overlain with a keratin plug. Compare with Figures 21, 22, and 23. Hematoxylin and eosin X 4



Figure 7.

Figure 8. Mitotic pattern in mouse interfollicular interscapular epidermis during the first 78 days of benzpyrene carcinogenesis. A single painting of benzpyrene in acetone was administered twice weekly. A six-hour mitotic rate was recorded at weekly intervals. The heavy black line represents the average mitotic rate. The area between the two fine black lines indicates the variation between lowest and highest rates.

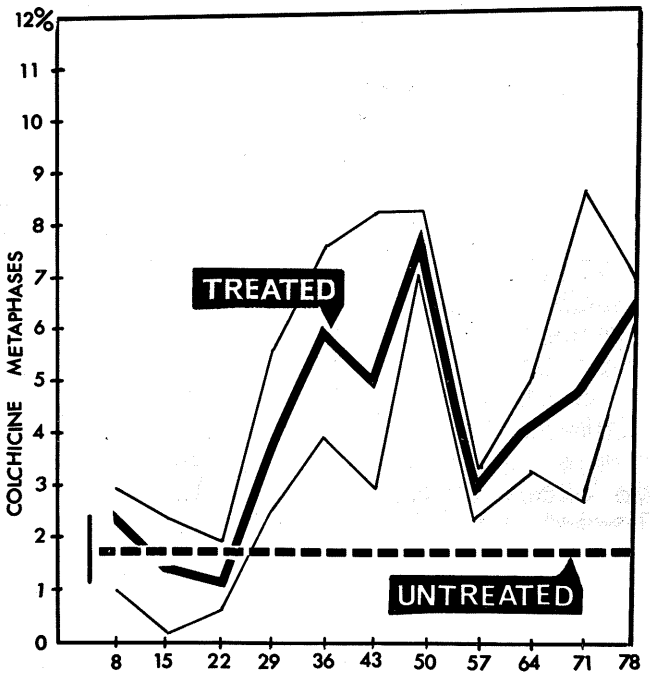
12%
11
10
9
8
7
6
5
4
3
2
1
0
COLCHICINE METAPHASES
TREATED
UNTREATED
8
15
22
29
36
43
50
57
64
71
78

Figure 8.

The lag period occurs during the first three weeks. The mitotic rates remain within normal levels. There is however a slight depression below normal at the second week, but while this difference was not statistically significant ($0.1 < P < 0.2$), the level of probability would however warrant a further collection of data.

The period of progressive increase in mitosis that follows the lag reaches a peak at 50 days. During this increase there is great variation in mitotic incidence among the animals.

From 50 to 57 days the mitotic rates fall off, indicating a depression in the precentage of cells entering mitosis, the lowest value almost approaches the normal level.

The terminal stage is characterized by a progressive recovery in mitosis with marked variability in individual response, especially after 71 days (20 paintings).

(b) II: Hair-follicular epithelium. The purpose of this experiment was to determine the mitotic pattern of a developing epidermal tumor of mouse skin. While the objective is the same as that of part I, this experiment differs in technique three ways: (1) the amount of carcinogen administered was increased as was the length of the experiment so as to increase the tumor yield, (2) colchicine metaphases were counted in the epithelium of the hyperplastic hair follicles and not in the interfollicular epidermis, and (3)

mitotic rates were not correlated with the number of paintings as was done in part I.

The hair follicular epithelium was studied because during the examination of the tissue sections it appeared that the papillomas were arising from the hair follicles. Moreover, Ghadially (1958; 1959; 1961) has presented evidence that the most common skin tumor of laboratory mammals arises from hair follicular epithelium.

The number of paintings were disregarded since mouse skin does not appear to react uniformly to equal carcinogenic treatment. This is illustrated in Fig. 9 which shows papilloma development in two mice which had received an equal number of paintings. Thus, in recording mitotic rates under the conditions of this experiment, it was not valid to assume that skin which had received a greater number of paintings was of necessity further advanced in the neoplastic process than skin which had received fewer paintings.

To ascertain the sequential development of papillomas, each tissue section was examined and compared to the photographs and descriptions in Ghadially's (1958; 1959; 1961) publications. Ghadially described two morphologically different papillomas, the Type 1 and Type 2 keratoacanthomas. Both types were observed in this experiment. The Type 1 papilloma was more common and thus, only the mitotic pattern during its histogenesis will be presented. The mitotic rates of the

Figure 9. Gross appearance of induced epidermal papillomas, showing the unequal response in two Swiss mice which had received an equal number of benzpyrene paintings.



Figure 9.

mature Type 2 lesion as well as the rates of carcinomas will be given.

(i) Histogenesis. While the life history of a Type 1 papilloma is a dynamic and apparently a morphologically continuous process from inception to maturation and finally regression, three stages were recognized in its histogenesis so as to facilitate a description and discussion of the mitotic activity of the developing tumor.

Ghadially (1961) considered the first stage in the evolution of the tumor to involve a thickening and elongation of the walls of adjacent hair follicles. In the following discussion an attempt was made to place the first stage further back in time prior to the hyperplasia of the follicles.

The germinitive region or "developmental bed" of the tumor appears to be characterized by an area of skin completely or partially denuded of hair follicles (Fig. 10). The epithelium consists of a hyperplastic covering epidermis and a follicular epithelium of degenerating or resting follicles. While the covering epidermis is five or six cell layers thick the follicular epithelium consists of only three or four layers of small cells enclosing a central cyst, loosely filled with a remnant of the hair shaft or strands of keratin.

The next stage (stage 2) of development involves (1) the appearance of proliferative foci in the basal layer of the covering epidermis and in the base of the resting follicles,

(2) the downgrowth of columns of cells from these foci (Fig. 11).

Stage 3 is characterized by an increased hyperplasia of the downgrowths and their fusion to form and irregular mass of follicular cells surrounding abortive hair shafts (Fig. 12, 13, 14).

The mature lesion (Fig. 15) results from the gradual extension of the hyperplastic follicles down into the depths of the dermis. The long papillary processes constitute the epithelial walls of the modified hair follicles. Between them are columns of keratin representing hair shafts. The keratin accumulates on the surface of the papilloma.

(ii) Mitotic rates. The mitotic rates of the hair follicular epithelium during the three stages of development are shown in Table VIII. The rate of stage 1 was taken from the covering epidermis.

The colchicine technique, in addition to providing an estimate of the percentage of dividing cells per unit time, also facilitates an identification of foci of proliferation. For example in stage 1, mitosis were scattered in the basal layer of the covering epidermis, while in stage 2, isolated foci began to appear in this layer. The down growths of the epithelial columns from these foci or from the base of the telogen follicles were "alive" with colchicine metaphases.

Figure 10. Stage 1 of keratoacanthoma Type 1 histogenesis. The "developmental bed" consists of an area of skin completely or partially devoid of hair follicles. At the right is a resting or telogen follicle. The covering epidermis is five or six cell layers thick. Hematoxylin and eosin X 16

Figure 11. Stage 2 of keratoacanthoma Type 1 histogenesis. Proliferative foci appear in the basal layer of the covering epidermis and in the base of the resting follicles. The foci enlarge to form downward growing columns of epithelial cells. Hematoxylin and eosin. X 16

Figure 12. Stage 3 of keratoacanthoma Type 1 histogenesis. The downgrowths become hyperplastic and irregular in outline. Note the hyperplastic epithelium surrounding the follicular lumen. Hematoxylin and eosin X 16

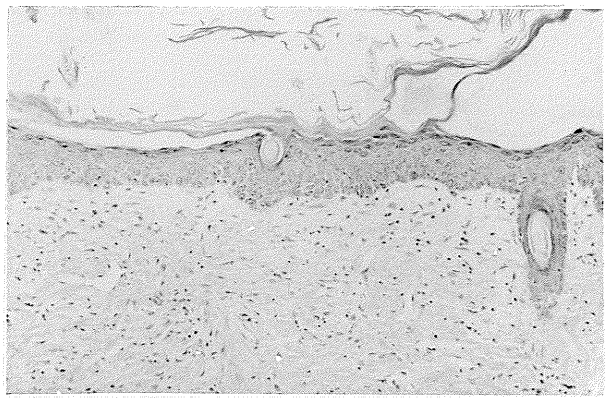

Figure 10.

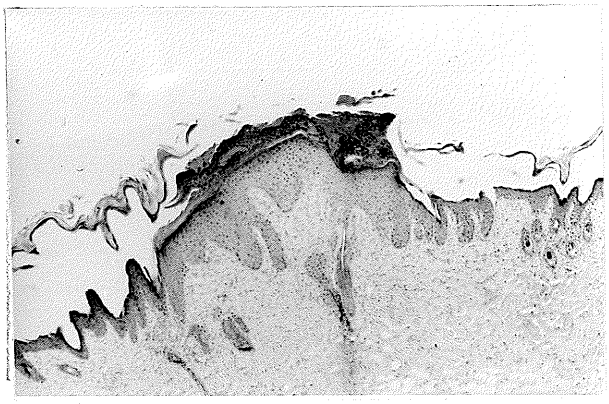

Figure 11.



Figure 12.

Figure 13. Stage 3 of keratoacanthoma Type 1 histogenesis--similar to Figure 12 but showing a folding of the hyperplastic epithelia. Hematoxylin and eosin X 16

Figure 14. Stage 3 of keratoacanthoma Type 1 histogenesis at a later time than Figures 12 and 13. The downgrowths have fused forming an irregular mass. Note the hyperplastic areas at the base of the three central aborted hair follicles. Hematoxylin and eosin X 16

Figure 15. Mature keratoacanthoma Type 1. The long papillary processes represent the hyperplastic walls of the hair follicles. Between the processes are columns of keratin representing abortive hair shafts. The lesion extends down to the panniculus carnosus. Hematoxylin and eosin X 4

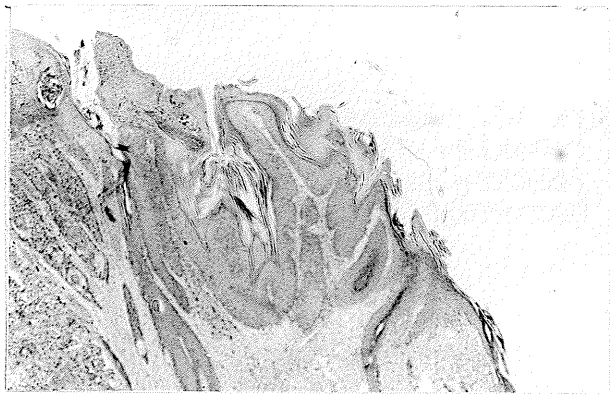

Figure 13.

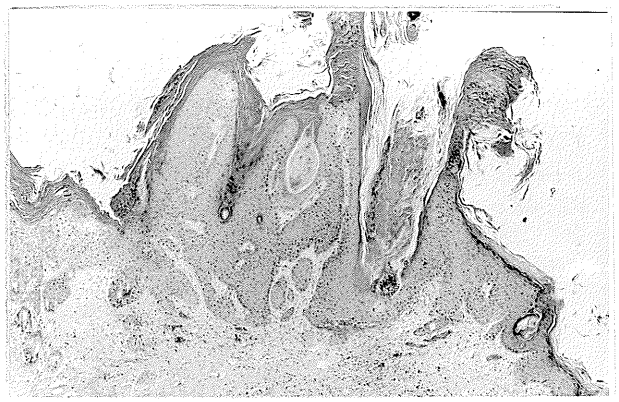

Figure 14.

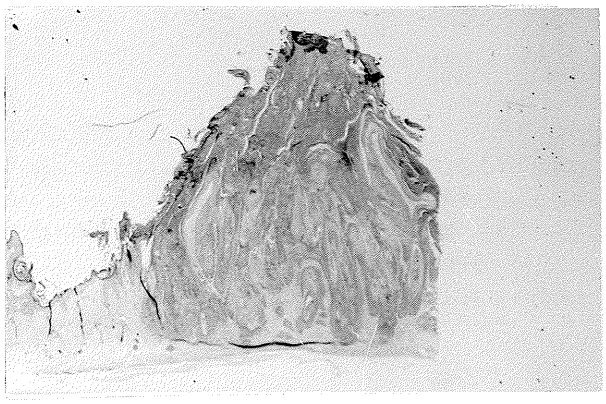

Figure 15.

TABLE VIII

MITOTIC RATES DURING BENZPYRENE EPIDERMAL CARCINOGENESIS
II: HAIR-FOLLICULAR EPITHELIUM

Developmental Stage	Figure No.	Nuclei No.	Metaphase No.	Per cent Metaphase	
	10	2027	74	3.65	
		2117	78	3.68	
1		2020	91	4.50	4.33 ± 0.76
		2024	111	5.48	
	11	2036	177	8.68	
		2058	187	9.07	
2		2019	189	9.36	9.15 ± 0.36
		2022	192	9.49	
		2059	115	5.59	
	13	2051	115	5.60	
3	14	2036	121	5.94	5.96 ± 0.52
	12	2042	137	6.71	

In stage 3, where the columns became confluent, the basal layers of the individual columns were diminished with a corresponding decrease in colchicine metaphases. Table VIII indicates that the downward extension of the epithelial columns is marked by a sharp increase in mitotic activity, more than double that of the covering epidermis. When the downgrowths unite, the mitotic rate also falls off. This decline may be correlated with a decrease in basal surface area, an increase in prickle cells and keratin formation.

A study of the mitotic rates of the remainder of the life history of Type 1 keratoacanthomas was complicated by the collection of an insufficient number of sections representing the mature form as well as the terminal stages of regression.

Only one mature Type 1 tumor was found (Fig. 15). It had a mitotic rate of 5.49 per cent which was similar to that of stage 3.

One path of regression involved the progressive pushing upward of the tumor and an undercutting of it by epithelial columns from the adjacent hair follicles (Fig. 16, 17). These columns eventually resurface the skin at the site of regression, (Fig. 18) to leave a three or four cell layered epidermis. The mitotic rates of four Type 1 tumors at this stage of regression were 3.07, 3.11, 3.42, and 3.50. The average mitotic rate was 3.27 per cent.

Figure 16. Regression of keratoacanthoma Type 1. The tumor is progressively pushed upwards to the surface. Hematoxylin and eosin X 10

Figure 17. Regression of keratoacanthoma Type 1, showing a higher power view of the base of the tumor (Figure 16). The epidermis is resurfaced from peripheral hair follicles. Hematoxylin and eosin X 16

Figure 18. Resurfaced hyperplastic epidermis at the regression site of a Type 1 keratoacanthoma. Hematoxylin and eosin X 16

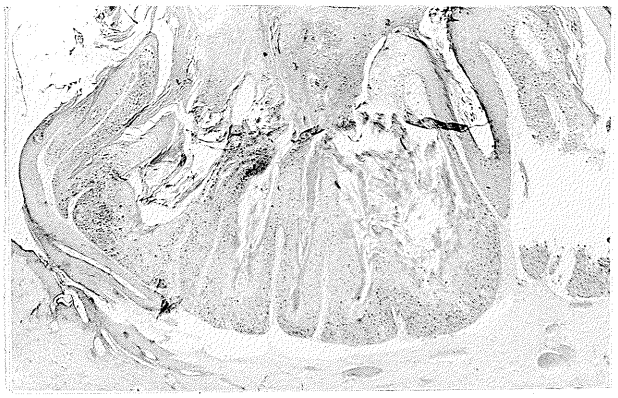

Figure 16.



Figure 17.

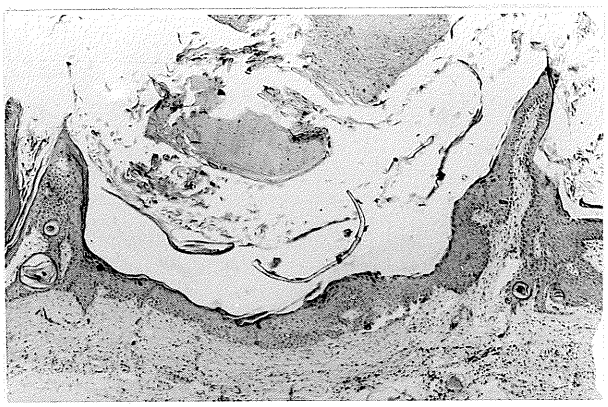

Figure 18.

Another path of regression apparently leads to a stalked branching form which protrudes from the surface (Fig. 19). Ghadially (1961) has called this tumor a "secondary papilloma." He considers it to arise from an exfoliation of the Type 1 keratoacanthoma and to be the most common path of regression in mouse skin. He states, "the so-called papillomas produced in this animal by the action of carcinogens are, in fact, the result of early exfoliation of Type 1 keratoacanthomas. . . ."

While the regression of the "secondary papilloma" was evident (Fig. 20) it was not possible to confirm or deny Ghadially's suggestion that it evolved from a Type 1 keratoacanthoma because of an insufficient number of these tumors. Only two "secondary papillomas" were observed and in both the mitotic rates were comparatively high for a regressing tumor (11.47 and 11.54 per cent).

On the basis of Ghadially's (1958; 1959) descriptions and illustrations, three papillomas were classified as Type 2 keratoacanthomas. With respect to their histology and mitotic rates they are categorized here into two subtypes.

In one type (Fig. 21, 22) the papillary arrangement was orderly and resembled that of Type 1 keratoacanthomas. The basement membrane was intact, there was no evidence of invasion and the vertical differentiation from the basal layer was maintained. More keratin was present than in the other subtype. Two of the three Type 2 keratoacanthomas presented

Figure 19. Stalked epidermal papilloma. Note the hyperplastic follicular epithelium surrounding the follicle lumen. The abortive hair shafts are represented by keratin lamellae. Hematoxylin and eosin X 10

Figure 20. Sequestered stalked papilloma. Hematoxylin and eosin X 10



Figure 19.

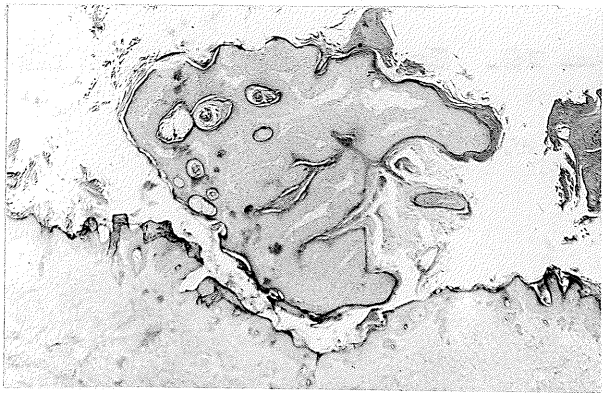

Figure 20.

this histology. Their mitotic rates were 4.58 and 5.11 per cent.

In the other subtype (Fig. 7, 23) the papillae were disorganized, the cells were pleomorphic and loosely arranged, tending to become separated from the papillae and invade the adjacent tissue. Two Type 2 keratoacanthomas were so classified, one from this experiment, the other from part I. The mitotic rates were comparatively high, being 12.00 and 10.00 per cent, respectively.

Two tumors were classified as epidermoid carcinomas, the principal criterion for malignancy was invasion of the panniculus carnosus (Fig. 24). The carcinomas were found co-existent with papillomas and appeared to have been derived from their cells (Fig. 25). The mitotic rates of the two carcinomas were 15.92 and 16.13 per cent; this is higher than the rate of papillomas, approximately 2.5 times higher than Type 1 keratoacanthomas and 3 times higher than one of the subtypes of Type 2 keratoacanthoma. The mitotic rate of the other subtype approaches the rate of the carcinomas.

3. EXPERIMENT 3

The purpose of this experiment was to estimate (1) the daily mitotic rate and (2) the doubling time of the alveologenic tumor of the mouse.

To ensure that each animal would yield a sufficiently

Figure 21. Keratoacanthoma, Type 2, subtype A. Subtype A is similar to that of B (Fig. 7) in that they form a crater or cystic lesion in the skin. They differ in histology of the papillae as well as mitotic rates. Hematoxylin and eosin X 6

Figure 22. A high power view of the papillae of keratoacanthoma Type 2, subtype A. The papillae are orderly, the basement membrane is intact; there is no evidence of invasion and the vertical differentiation from the basal layer is maintained. Hematoxylin and eosin X 64

Figure 23. A high power view of the papillae of keratoacanthoma Type 2, subtype B. The papillae are disorganized, the cells are pleomorphic, loosely arranged and tending to invade the adjacent tissue. Hematoxylin and eosin X 64

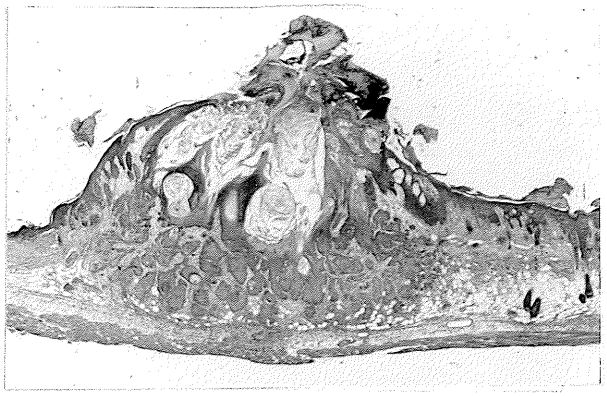

Figure 21.

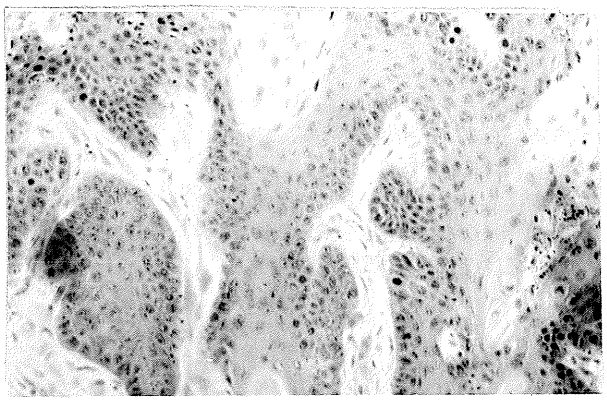

Figure 22.

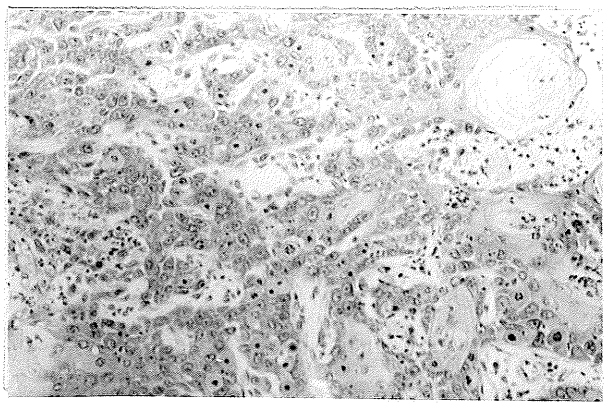

Figure 23.

Figure 24. Epidermoid carcinoma of mouse skin showing invasion of the panniculus carnosus. Note epithelial pearls. Hematoxylin and eosin. X 64

Figure 25. Carcinomatous infiltration from the base of a regressing Type 1 keratoacanthoma. Hematoxylin and eosin X 10

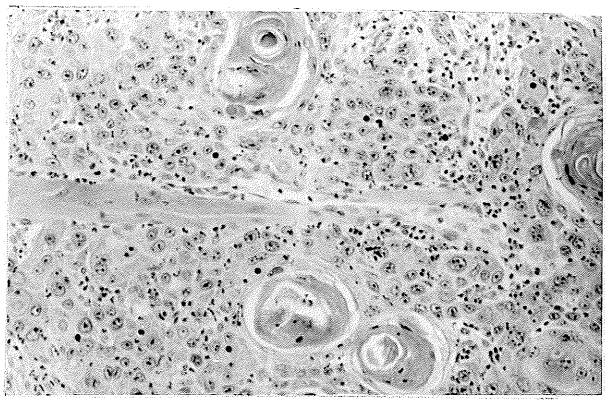

Figure 24.

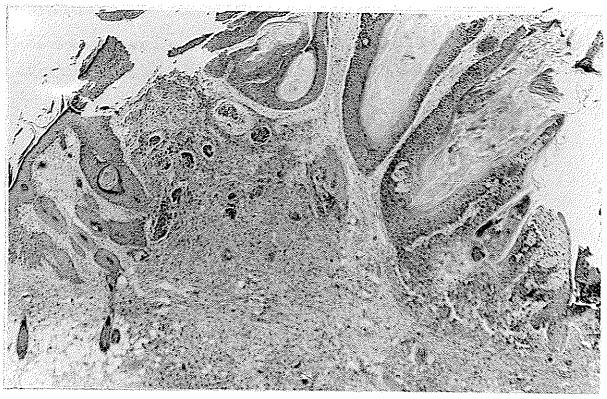

Figure 25.

large number of tumors, each mouse received a single intraperitoneal injection of urethane 209 days prior to sacrifice.

Sixteen mice were subdivided into four groups, each group consisting of four animals. Each mouse received a single subcutaneous injection of colchicine. The first group was injected at 10 a.m., the second at 4 p.m., the third at 10 p.m., and the fourth group at 4 a.m. The animals were sacrificed six hours after colchicine injection.

At the time of sacrifice multiple lung tumors had developed in each mouse as evidenced by the presence of macroscopically visible pearly white nodules projecting from the pleural surface. Lung tissue bearing the nodules was randomly removed from all lobes.

(a) Histology. All of the 36 tumors that were microscopically examined had the basic adenomatous pattern of the alveologenic tumor of the mouse. One of the neoplasms, however, had certain distinct features which set it apart from the others (Fig. 26). It was darker staining and in close association with a bronchiole. The bronchial epithelium, however was not hyperplastic nor was there an increase in metaphase figures. The cells of the tumor were larger, the cytoplasm more eosinophilic, the nuclei larger and the nuclear borders more pronounced. In one area of the neoplasm, a papillary extension was occluding the lumen of a bronchiole.

Figure 26. Papillary adenocarcinoma of mouse lung. Showing apparent invasion into a bronchial lumen. Hematoxylin and eosin X 10

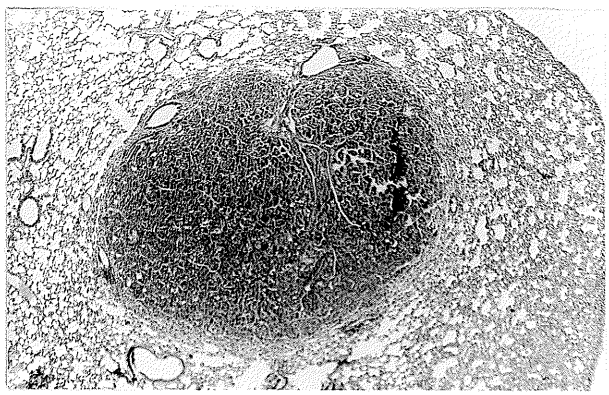

Figure 26.

There was no invasion into the surrounding lung tissue, rather the tumor presented a definite round circumscribed mass. Unfortunately a search for metastases was not undertaken. Cloudman (1941) has described a pulmonary tumor of the mouse with a similar histology to which he gave the name "papillary adenocarcinoma." This term will be used here in reference to the tumor.

The histology of the remaining 35 tumors were similar and agreed with the classic description for the histology of the common alveologenic tumor of the mouse lung (Tyzzer, 1907; 1909; Slye et al., 1914; Cloudman, 1941; Shimkin, 1955; Stewart, 1959) (Fig. 27). There is, thus no need to repeat here the histology of this tumor. In contradistinction to the papillary adenocarcinoma and to facilitate a discussion of their mitotic rates they will be referred to as "papillary adenomas."

(b) Mitotic rates. The mitotic rates for the four, six-hour periods is shown in Table IX. The table indicates that the mitotic rate of the "papillary adenoma" is not constant for a six-hour period, but appears to vary with the time of day. The average mitotic rate for the 10 a.m. to 4 p.m. interval was 0.30 ($\pm$ 0.08). During the next six hours the average mitotic rate fell to approximately 0.10 per cent and remained at this level for the following six-hour period.

Figure 27. Papillary adenoma of mouse lung.
Hematoxylin and eosin X 16

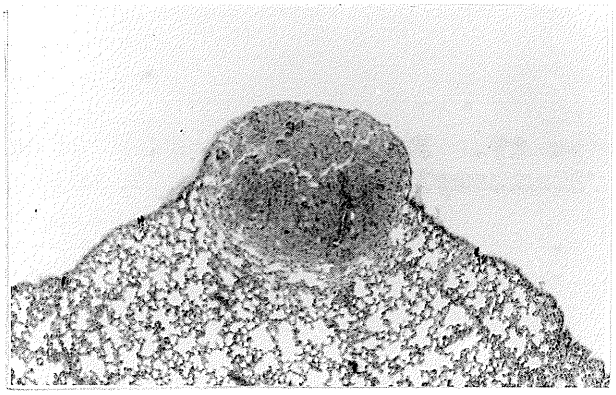

Figure 27.

TABLE IX

DAILY MITOTIC RATE OF MOUSE ALVEOLOGENIC PAPILLARY ADENOMAS

Time of Day	Nuclei No.	Metaphase No.	Per cent of Metaphase	
10 A.M.-4 P.M.	2051	8	0.39	
	2112	5	0.24	
	2040	6	0.29	0.30 ± 0.08
	2048	6	0.29	
4 P.M.-10 P.M.	2165	3	0.14	
	2094	2	0.09	
	2079	2	0.10	0.09 ± 0.05
	2064	1	0.05	
10 P.M.-4 A.M.	2065	4	0.19	
	2053	2	0.10	
	2048	2	0.10	0.12 ± 0.08
	2060	3	0.14	
4 A.M.-10 A.M.	2037	22	1.08	
	2053	1	0.05	
	2077	20	0.96	0.60 ± 0.48
	2038	6	0.29	

Daily mitotic rate = 1.11 ± 0.69

This decrease (0.30 to 0.10) from late afternoon to early morning was statistically significant ($0.001 < P < 0.01$). The average mitotic rate for the last interval (4 a.m. to 10 a.m.) was 0.60 per cent with a relatively high standard deviation of 0.48.

Summation of the four average mitotic rates gives a value of 1.11 which is an estimate of the percentage of tumor cells dividing in one day.

Knowledge of the daily rate of mitosis allows for an estimate to be made of the doubling time of a tumor. The doubling time is defined as the time required for the doubling of the number of cells, (Kisieleski et al., 1961). The doubling time, based on the colchicine technique is calculated by assuming that if 1.11 per cent of the papillary adenoma cells enter mitosis over a 24 hour period, the number of cells equal to 100 per cent will require $24/1.11 \times 100 = 2180$ hours or approximately 91 days. This figure indicates that for every 91 days the cell number of the tumor will become doubled. The validity of this value will be examined in the discussion.

The mitotic rate of the papillary adenocarcinoma for a six-hour period (4 a.m. to 10 a.m.) was 1.76 which is almost twice the daily mitotic rate of the papillary adenoma. Comparison of the adenocarcinoma's rate with that of a papillary adenoma (0.29) which was growing in the same lobe and whose rate was obtained for the same interval, indicated that the

adenocarcinoma produced approximately six times as many cells than the adenoma in a six-hour period.

There were two papillary adenomas observed in the 4 a.m. to 10 a.m. period which had a mitotic rate of approximately one per cent, which is 20 times greater than the lowest mitotic rate recorded for a papillary adenoma. The significance of this range will be examined in the discussion.

4. EXPERIMENT NO. 4

The purpose of this experiment was to determine whether pulmonary tumors would arise when urethane crystals were applied directly to pulmonary tissue.

In this experiment, large pieces of lung tissue were removed and in vitro, wrapped about a few crystals of urethane and implanted into the subcutaneous tissue of the ventral body wall of homologous hosts. Control animals received lung grafts only.

At various intervals, areas of the skin and fascia into which the grafts had been inserted, were removed and examined microscopically, for the presence of tumors.

No tumors, adenomas or other types were found in those lung implants that survived and became established. Of 28 grafts implanted, 19 survived while the remainder had apparently failed to become vascularized.

(a) Histology. The microscopic appearance of both

control and experimental grafts were similar (Fig. 28, 29). The alveolar spaces were collapsed and there was an increase in connective tissue. There were no severe inflammatory reactions or areas of extensive necrosis, however, in many of the grafts treated with urethane, the bronchi were dilated and filled with cellular exudate (Fig. 30).

Only one or two colchicine metaphases were found in both experimental and control grafts suggesting that there is little or no cell division in grafted lung.

5. EXPERIMENT NO. 5

The purpose of this experiment was to confirm Horning's technique (1947, 1950) of inducing bronchogenic tumors and to record the mitotic rates of the neoplasms.

Lung tissue was removed, directly impregnated with crystals of methylcholanthrene and transplanted to the subcutaneous tissue of homologous mice. For controls, grafts of lung tissue not containing methylcholanthrene were transplanted, and to another series of mice methylcholanthrene crystals were directly deposited into the subcutaneous tissue.

(a) Subcutaneous implants of methylcholanthrene. Of the seven mice which received a single subcutaneous implant of methylcholanthrene crystals, a fibrosarcoma was microscopically observed in one animal which was sacrificed 211 days after treatment (Fig. 31). In the remaining animals there was

Figure 28. Mouse lung graft not containing a carcinogen. The lung was transplanted into subcutaneous mouse tissue 212 days prior to sacrifice. The alveolar spaces are collapsed, colchicine metaphases were absent and there was no evidence of neoplastic foci. Hematoxylin and eosin X 10

Figure 29. Mouse lung graft impregnated with urethane 343 days prior to sacrifice. The graft was transplanted to subcutaneous mouse tissue. Neoplastic foci were not evident. Hematoxylin and eosin X 10

Figure 30. Mouse lung graft impregnated with urethane 343 days prior to sacrifice. Showing dilated bronchi occluded with cellular exudate. Neoplastic foci were not observed. Hematoxylin and eosin X 10

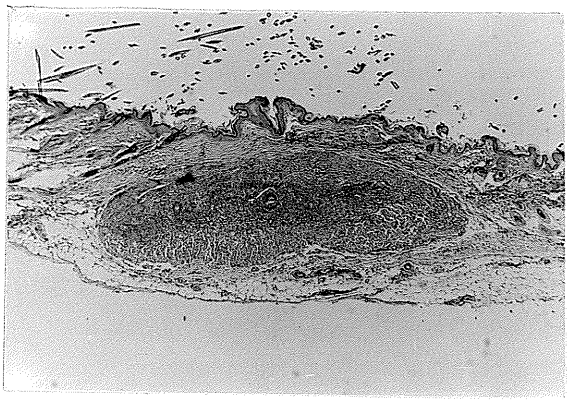

Figure 28.

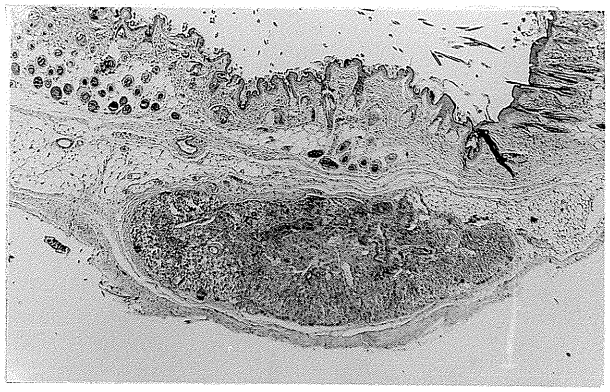

Figure 29.

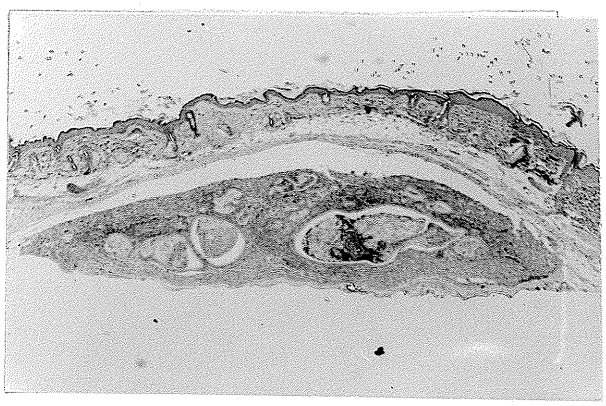

Figure 30.

Figure 31. Fibrosarcoma resulting from deposition of methylcholanthrene crystals into mouse subcutaneous tissue, 211 days prior to sacrifice. Hematoxylin and eosin X 64

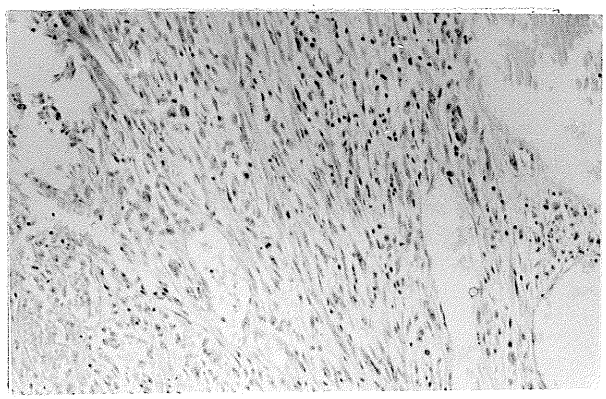

Figure 31.

an inflammatory reaction and an overgrowth of connective tissue in the vicinity of the deposition site.

(b) Subcutaneous implants of lung only. Of the five mice which received a single implant of lung tissue not impregnated with methylcholanthrene, three of the implants could not be found and apparently were reabsorbed due to vascular failure. Two of the lungs had survived to 115 and 212 days after implantation (Fig. 28). No neoplastic cell populations were observed in the surviving lungs.

(c) Lung tissue combined with methylcholanthrene. At sacrifice, 109 days after implantation, tumors were recovered from 18 of the 25 mice. One tumor was taken from each mouse. The tumors were classified as epidermoid carcinomas (9), fibrosarcomas (4), undifferenciated carcinomas (4) and one adenocarcinoma. Since no ciliated cells could be found at the periphery or within the fibrosarcomas it is believed this tumor arose from the subcutaneous tissue and was therefore excluded from study.

Using Kreyberg's (1962) criteria for the classification of lung tumors, epidermoid carcinomas were recognized by their slight keratinization, definite stratification and intercellular bridges (Fig. 32). The intercellular bridges were not always evident. Microscopically, the tumors had a branching papillary architecture (Fig. 33).

The adenocarcinoma had a characteristic gland formation with pools of mucin. Ciliated cuboidal as well as goblet cells lined the mucin distended acini (Fig. 34).

The undifferentiated cancers were densely cellular with no obvious stroma. The pleomorphic cell population consisted of cells with large hypochromatic nuclei and those with small hyperchromatic nuclei.

(1) Mitotic rates. Mitotic counts were taken from the epidermoid carcinomas and the single adenocarcinoma. The mitotic rates of the epidermoid carcinomas are shown in Table X. The average mitotic rate was 5.11 with a range from three to six per cent. Unfortunately an insufficient number of epidermoid carcinomas were recovered in order to determine the presence or absence of a diurnal rhythm. The adenocarcinoma had a mitotic rate for a six-hour period (4 p.m. to 10 p.m.) of 2.06 per cent.

Figure 32. High power of epidermoid carcinoma which had developed in a lung graft impregnated with methylcholanthrene crystals 109 days prior to sacrifice. Note the stratification of the papillae and keratinization. Hematoxylin and eosin X 64

Figure 33. Low power of epidermoid carcinoma shown in Figure 32. Note the branching papillary arrangement. Hematoxylin and eosin X 10

Figure 34. Adenocarcinoma which had developed in a lung graft impregnated with methylcholanthrene crystals 109 days prior to sacrifice. Note the acini distended with mucin. Hematoxylin and eosin X 64

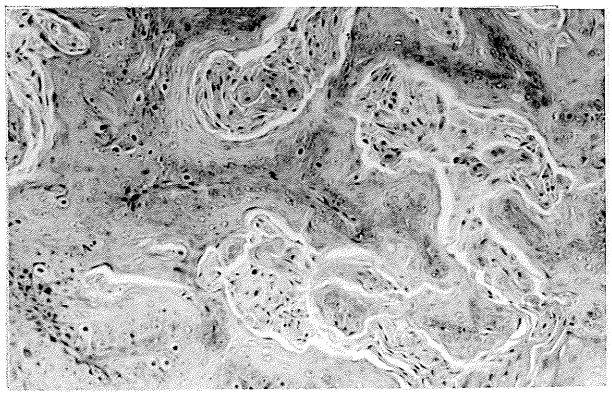

Figure 32.

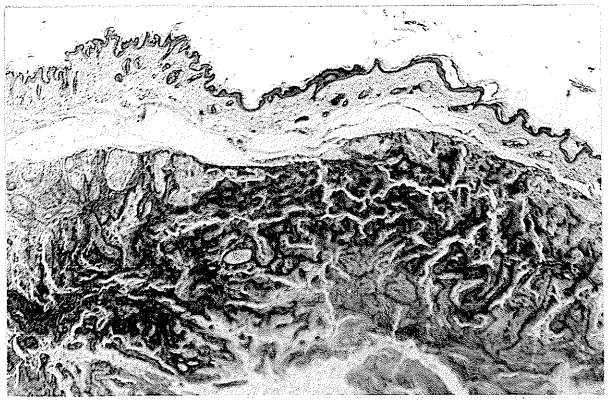

Figure 33.

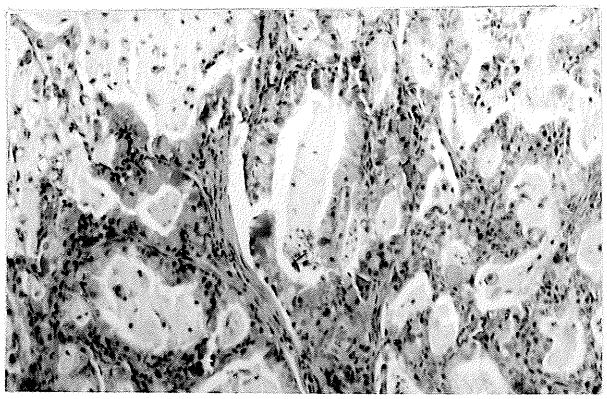

Figure 34.

TABLE X

MITOTIC RATES OF LUNG EPIDERMOID CARCINOMAS

Time of Day	Nuclei No.	Metaphase No.	Per cent of Metaphase	
10 A.M.-4 P.M.	2050	65	3.17	
10 A.M.-4 P.M.	2024	74	3.66	
4 P.M.-10 P.M.	2056	76	3.70	
10 A.M.-4 P.M.	2030	86	4.24	
10 P.M.-4 A.M.	2039	87	4.27	4.55 ± 0.96
10 P.M.-4 A.M.	2051	101	4.92	
10 P.M.-4 A.M.	2020	107	5.30	
10 P.M.-4 A.M.	2034	113	5.57	
4 A.M.-10 A.M.	2183	133	6.09	

CHAPTER V

DISCUSSION

1. EXPERIMENT NO. 1

(a) I: Ear epidermis. Comparison of the daily mitotic rates of treated and untreated ear epidermis indicates that a single painting of an 0.3 per cent solution of benzpyrene in acetone depresses the daily production of new cells by approximately 34 per cent. This inhibition of mitosis occurs largely from 12 hours onward after the painting. During the first 12 hours after treatment the carcinogen has little effect in stimulating or depressing mitosis.

Heller and Cooper (1944) reported a similar experiment except they applied a 0.6 per cent solution of methylcholanthrene in benzene and recorded the mitotic indices at 6, 12, 18 and 24 hours after a single painting to the ears of mice.

The pattern of mitotic activity they reported differs from the one observed here. At 6 and 12 hours after painting the mitotic activity was similar in experimental and control groups. This observation was confirmed in the present experiment. At 18 hours, however, the mitotic index rose above the normal level and remained above it for the duration of the experiment. This observation could not be supported by the present data.

While the discrepancy may have been due to the use of different carcinogens, solvents and concentrations, it is unlikely that they were solely responsible. The difference is

perhaps largely due to Heller and Cooper's failure to control the mitotic duration.

The increase in mitotic figures which they observed at 18 and 24 hours after treatment may not have been due to an acceleration of mitosis, which they believed to have occurred, but more to a lengthening of the mitotic duration, which would result in the persistence of mitotic figures in the tissue. That this may be a valid interpretation of their data is suggested by the results of the present experiment in which the colchicine technique takes into account any variation in mitotic duration that may occur. A prolonging of the mitotic duration would be represented in the colchicine results as a depression or inhibition of cells entering mitosis and not as an increase. This result was observed.

The present experiment suggests that Heller and Cooper's assumption that a carcinogen stimulates mitosis 12 hours onward to 24 hours following a single painting, may be unwarranted.

Evensen (Iversen and Evensen, 1962) applied a single application of a 1.0 per cent solution of methylcholanthrene in benzene to the skin of hairless mice and reported that following the injection of colcemid, the mitotic rate after 24 hours was three times that of untreated skin.

Evensen appears to have misinterpreted the colchicine technique. He refers to the "per cent of mitotic figures"

after 4 or 6 hours after colcemid injection as the mitotic index or mitotic count. This value according to the colchicine technique is referred to as the mitotic rate. The author calculates the mitotic rate according to the formula mitotic index/mitotic duration. While the formula is valid, Evensen however has inserted the value for the colchicine mitotic rate in the place of the mitotic index.

Examination of his tables (No. 34, 42) suggests that there is no difference in the mitotic rates of treated and untreated skin 24 hours after a single painting.

Iversen and Evensen's (1962) theory of cell population shifts and the kinetics of epidermal reaction to carcinogens may require re-examination.

The significance of the mitotic pattern reported in the present experiment, that is, an initial unchanged rate of mitosis followed by a depression is difficult to interpret. This pattern may be unrelated to the carcinogenic process.

It is known, however, that a single painting of a carcinogen is sufficient to produce skin tumors in mice (Mider and Morton, 1939; Cramer and Stowell, 1943). Moreover, McCarter (1959) has shown that carcinogenic changes do occur within the first few hours following a single application of benzpyrene to mouse skin. These observations do not necessarily imply that the mitotic reponse following carcinogenic treatment is a reflection of the neoplastic changes occurring in the tissue. It may well be, that the cellular proliferation is a

generalized non-specific response of the tissue that can also be invoked by non-carcinogens. Future experiments will be required to determine whether carcinogens induce a specific mitotic pattern that is distinguishable from that of non-carcinogenic agents.

Attempts to correlate mitotic response with levels of protein and DNA bound carcinogens (Miller, 1951; Brookes and Lawley, 1964) may be in order.

(b) II: Interscapular epidermis. Chase et al., (1953) reported that the thickness of mouse interfollicular epidermis and the incidence of mitotic figures varied with the stages of the hair cycle. This observation could not be confirmed by Klinken-Rasmussen (1954). The results of this experiment support the observation of Chase et al., (1953) that the hair follicular cycle modifies interfollicular mitotic activity. The results also suggest that the variation in mitotic rates between the animals in both control and experimental groups can be attributed to the hair cycle.

In the control group, an analysis of variance revealed that there was no significant difference between the four means. This suggests that a diurnal rhythm of mitosis was absent in interscapular epidermis. This conclusion is not compatible with the results of Bullough (1948), nor are they in agreement with the widely demonstrated occurrence of such

a rhythm in a variety of mouse and rat tissues.

The failure to observe such a day and night variation in mitosis may be due to (1) an actual absence of such a rhythm in interscapular epidermis or (2) the rhythm, although present, was obscured by individual variation resulting from the growth of the hair follicles. Of the two, the second possibly is favored (Tables V, VI).

It may be maintained however, that this influence is not of sufficient magnitude to obscure a diurnal rhythm since Bullough (1948) observed such a phenomena, yet the hair cycle in his experiment was not controlled. The possibility that other factors such as light, temperature, diet and handling were responsible tends to be ruled out since the mice in part I were treated similarly, yet a diurnal rhythm was observed in the ear epidermis.

The interval in the control group from 4 p.m. to 10 p.m. had a smaller coefficient of variation than the interval from 10 a.m. to 4 p.m. In the former, the smaller value can be correlated with a grouping of similar stages of the hair cycle, while the latter can be correlated with a greater variation in the stages of the cycle.

It is believed that if in the present experiment all animals had been in the same stage of the hair cycle, the variation of mitotic rates would have been considerably reduced, and that statistical analysis would have indicated a

diurnal pattern in cell division.

In the experimental group a diurnal pattern was not observed, nor was there a statistically significant difference between the daily rate of mitosis in treated and untreated ears. The absence of these phenomena may have been due to the carcinogenic treatment and/or the growth of the hair follicles. The hair cycle is believed to be largely responsible.

To isolate the effect of a carcinogen on the incidence of mitosis in the epidermis, the time of day as well as the hair cycle must be the same in treated and untreated animals. During the planning of this experiment the time of day was considered but not the hair cycle. As a result, each six-hour interval was represented by four unselected animals in different stages of the hair cycle. Since few treated and untreated mice were alike for both factors of time and hair cycle, conclusions regarding the early effect of benzpyrene on mitotic activity of the interscapular epidermis can not be made. Moreover, failure to control the hair cycle makes it impossible to separate from a given mitotic rate in the experimental animals, the percentage of cells that divided because of carcinogenic stimulation from the proportion of cells dividing due to the hair cycle.

The present experiment suffers from the failure to adequately control the hair cycle. As a result, it is not possible to arrive at a convincing answer as to whether a

single painting of benzpyrene accelerates or depresses mitotic activity in interscapular epidermis during the first 24 hours of its carcinogenesis.

Previous experiments measuring mitotic activity in which the growth of the hair was not considered may be open for re-evaluation.

2. EXPERIMENT NO. 2

(a) I: Interfollicular epidermis. The purpose of this experiment was to record the mitotic rates and pattern during the development of an epidermal tumor. Considering the histogenesis of papillomas from hair follicles (Ghadially, 1961; and part II of this experiment) it is unlikely that the mitotic rates of interfollicular epidermis are those of a developing papilloma. They may however, be a reflection in the interfollicular epidermis of the changes occurring in the hair follicles, or the rates may mirror the cellular or tissue changes leading to the establishment of malignant cells in the epidermis for morphological evidence has been put forward that carcinomas may develop from hyperplastic epidermis (Cramer and Stowell, 1942; Glücksmann, 1945; Siegler and Duran-Reynals, 1962).

If it assumed that (1) the malignant conversion may occur in interfollicular epidermis and (2) this conversion occurred during this experiment the mitotic rates may or may

not reflect the establishment of the cancerous cell. The mitotic pattern may be a non-specific reaction that occurs following carcinogenic as well as non-carcinogenic treatment. This, however, seems unlikely since the repeated painting of coal tar (Champy and Vasiliu, 1923), methylcholanthrene (Cooper and Reller, 1942) and now benzpyrene all result in the same basic pattern; arise in mitosis, a mid point depression, and a terminal increase.

One would like to know if this pattern is retained under various experimental conditions such as in tissue culture, in grafts free of dermal tissue, and following treatments with single and multiple paintings of different carcinogens. The role of the hair cycle requires investigation as does the histogenesis of carcinomas. The relationship of this pattern to the phenomena of "initiation" and "promotion" in the skin may be worthy of experimentation.

Until more is known of the characteristics of this pattern little can be said for its significance in relationship to the establishment of neoplastic foci.

(b) II: Hair-follicular epithelium. Ghadially (1958, 1959, 1961) in his comprehensive study of the role of the hair follicle in skin tumorigenesis has suggested that the keratoacanthoma is a modified or perverted hair follicle. It takes origin from a follicle and has a life cycle that parallels that of a normal hair follicle.

The results of the present experiment indirectly support this hypothesis. The histogenic study confirms Ghadially's observation that the keratoacanthoma Type 1 arises from the hair follicle and the mitotic rates suggest that the tumor mitotic activity parallels that of the normal follicle during its cycle of growth and regression.

As to the histogenesis of Type 1 papillomas, Ghadially (1961) believed that the first morphological evidence of their presence was a thickening of the walls of adjacent hair follicles. An attempt was made to carry this process further back in time to the regeneration of new follicles from the covering epidermis or from the resting follicles. It is during this attempt of the "primitive hair germ" to reform the hair follicle that the lack of mitotic restraint becomes evident and the walls of the follicles become hyperplastic and coalesce to form a tumor mass.

This suggestion is based on indirect morphological evidence for it could not be established whether these so-called "primitive hair germs" would have gone on to form the tumor or whether they would have formed a normal or slightly altered hair follicle, if the animal had been allowed to remain alive.

The pattern of mitotic activity during the histogenesis of the Type 1 keratoacanthoma suggests that this tumor arises from the hair follicle because the mitotic rates increase

during the early stages of formation, reach a peak in the mature established tumor and then decrease as the tumor regresses. This follows the pattern of mitosis in the normal hair follicle. The follicle grows down into the dermis accompanied by an increased mitotic activity, then becomes established in the dermis where hair production occurs. Mitosis increases in the hair bulb during the stage of hair production. It then declines as hair production ceases, and the follicle regresses. It is unfortunate that a control series in which the mitotic rates of follicular epithelium during a normal hair cycle were not available. It would be of interest to determine if the rates are higher, lower or similar. This clearly is the next step in studying the mitotic behavior of the tumor.

The estimation of the mitotic rates of the tumor during the remainder of its life history, that is, the stage of regression was made difficult by the collection of so few specimens.

During regression, at least along one path, the mitotic rate decreases from the mature tumor. In the stalked papilloma (Fig. 19, 20) the mitotic rate is high, being about six times that of a mature Type 1 keratoacanthoma. No explanation can be given here for this difference. The possibility may exist that some stalked papillomas arise simply from an upfolding of the developing tumor and not as Ghadially (1961) believed as an

exfoliation of the Type 1 neoplasm. In this study there was no morphological evidence to either support or deny the possibility, although an independent development of the stalked papilloma may be more in keeping with its relatively high mitotic rate than the exfoliation hypothesis suggested by Ghadially (1961). A collection of a large number of these tumors and a more detailed study of their histogenesis is required.

The mitotic rates of the keratoacanthoma, Type 2, vary with morphology. Two subtypes were recognized, on the basis of differences in morphology and behavior. One subtype was similar to the Type 1 keratoacanthoma while the other approached the carcinoma both in histology and mitotic rate. This does not imply that all carcinomas evolve from Type 2 keratoacanthomas, because carcinomatous infiltration was observed originating from the base of a regressing Type 1 keratoacanthoma (Fig. 25).

The mitotic rate of the carcinoma exceeds that of the papillomas by approximately three times.

It may be of interest to estimate the daily rate of mitosis, and the doubling time of the carcinoma. The estimate is based on only two tumors. The percentage of dividing cells for a six hour period of the two tumors was approximately 16 per cent. Assuming an absence of a diurnal rhythm, the daily mitotic rate would be 64 per cent (16 x 4). If a

diurnal rhythm was present, the daily mitotic rate would be slightly less. On the basis of the daily rate of mitosis, the doubling time is estimated as approximately 38 hours or 1.6 days. The method of calculating colchicine doubling times as well as a criticism of such values is presented in Experiment No. 3.

It may be of interest to compare the doubling time of the epidermoid carcinoma of the skin with those of other malignant tumors.

The doubling time of the epidermoid carcinoma is exceeded by that of Ehrlich ascites tumor cells (18 to 20 hours), (Baserga et al., 1963). The doubling time is similar to that of a transplanted Walker 256 carcinosarcoma (1.7 days), (Bertalanffy and Lau, 1962) and is less than the doubling times of the transplanted 1F16F rat fibrosarcoma (2.5 days), (Bertalanffy and Lau, 1962), the transplanted B16 mouse melanoma (2.8 days), (Bertalanffy and McAskill, 1964), the spontaneous mouse mammary gland carcinoma (8.5 days), Bertalanffy, 163a) and the methylcholanthrene induced rat fibrosarcoma (10.6 days), (Bertalanffy, 1963b).

3. EXPERIMENT NO. 3

The morphological results confirm the observations of Slye et al., (1914) and of Cloudman (1941) that the common pulmonary adenoma or alveologenic tumor of the mouse

consists of a series of neoplasms that extend from the benign to the malignant. Shimkin (1955) believed that the alveologenic tumor passes through a benign stage where it may be termed an adenoma. Later it may acquire the malignant characters of invasiveness and metastasis in addition to its unencapsulation and transplantability. At this stage it may be referred to as an adenocarcinoma. The "papillary adenocarcinoma" observed in this experiment may be considered as advancing toward malignancy while the "papillary adenomas" are relatively benign.

The mitotic rates indicate that the number of tumor cells which are added to the "papillary adenoma" are not constant for a given six-hour period. This variation in the rate of mitosis may be due to (1) a diurnal rhythm, or (2) a random selection of tumors which are morphologically alike but grow at different rates.

Both factors appear to be involved.

For purposes of discussion, the papillary adenomas will be subdivided into two types on the basis of their mitotic rates. Type A consists of all adenomas except the two in the 4 a.m. to 10 a.m. interval which have mitotic rates of one per cent and are referred to as Type B adenomas. The Type A adenomas have a mitotic rate range from 0.05 to 0.39 per cent.

The difference in mitotic rates between these two

classes of morphologically similar adenomas is greater than could be expected if a diurnal rhythm was operating.

Assuming malignancy is associated with progressive increase in mitosis, Type A tumors would be benign, Type 2, less so and the papillary adenocarcinoma as previously described would be close to malignancy. This change in mitotic activity of the alveologenic tumor during its lifetime is an example of a phenomena termed "progression." Foulds (1951) defined progression as "the advancement of a tumor by irreversible qualitative changes of one or more of its characters." While progression has been observed in other tumor systems this apparently is the first report of its occurrence in the alveologenic tumor of the mouse.

Type A adenomas have a considerable range of mitotic rate, extending from 0.05 to 0.39 per cent. This range is taken to indicate the existence of a diurnal rhythm, because in each of the first three six-hour intervals there is little variation between the individual tumors within each group. Secondly, in the first six-hour period (10 a.m. to 4 p.m.), the average mitotic rate was 0.30 per cent. It then declines for the next 12 hours to approximately 0.10 per cent, a decrease which is statistically significant. This depression at this time of day corresponds to a typical pattern of a dirunal rhythm.

The daily mitotic rate as shown in Table IX includes

both Type 1 and 2 adenomas and therefore can not be considered representative of either class. Because Type 1 tumors were more numerous, they have been selected as a base from which to compare the mitotic activity of neoplastic alveolar tissue with its normal counterpart.

Since the mitotic rate for normal mouse alveolar tissue was obtained for the 10 a.m. to 4 p.m. interval (Bertalanffy and Leblond, 1953), the same interval was chosen here. The average mitotic rate for this period was 0.30 ($\pm$ 0.08) per cent, compared to 1.13 ($\pm$ 0.20) in the normal tissue. This difference implies that in the early stages of the life of the alveologenic tumor the number of dividing cells is depressed below normal values. In the later stages the mitotic rate increases to reach the normal, while in the papillary adenocarcinoma, the rate exceeds the normal.

This observation, that a neoplastic cell population has fewer dividing cells than the parent tissue, was unexpected. Such an observation has apparently not been reported previously and it requires confirmation. Should similar findings result, it may indicate that the establishment of this neoplasm does not necessarily involve an increase in the rate of cell division. The tumor may increase in size in the early stages more to a failure of exfoliation of cells than to a rapid rate of mitosis. Perhaps, once a critical cell number is reached, the rate of division shifts to progressively higher

levels.

Comparison of the mitotic rate of the tumor cells with those of the alveolar cells involves the assumption that the neoplastic cells are converted alveolar cells. There is no direct evidence for this belief.

If the tumor cells are transformed mesodermal phagocytic alveolar cells it is difficult to appreciate the epithelial-like arrangement of the adenoma. Such an organization would suggest that the surface pulmonary or the bronchiolar rest cells become neoplastic. In the absence of morphological studies and mitotic rates of these cells little can be said in support of this hypothesis. Should future experimental evidence suggest that these epithelial cells are the source of the tumor, difficulty may arise in understanding the sarcomatous pattern the adenoma assumes when it metastases (Wells et al., 1941) or when it is serially transplanted to the subcutaneous tissue of mice of the same strain as the animal in which the primary carcinomatous tumor arose (Andervont, 1939a). While a number of theories have been proposed in an attempt to explain this sarcomatous change, "the mechanism and underlying causes, . . . cannot be explained satisfactorily" (Stewart, 1959).

Indirect support for the alveolar cells not being the source of the tumor is suggested by the results of Shimkin and Lorenz (1942) who gave intraperitoneal injections of

trypan blue prior to injection of dibenzanthracene. They found that the reticulo-endothelial blockade had no effect on tumor incidence. Grady and Stewart (1940) mentioned that some tumor cells contained pigment particles within their cytoplasm. It was not established if the pigment was the result of phagocytic activity or represented an endogenous metabolic product.

More extensive experiments based on the present concept of the structure of the alveolar tissue (Bertalanffy, 1964) combined with measurements of mitotic activity may be of value in suggesting some possible conclusions.

Because an insufficient number of Type 1 adenomas were collected the daily rate of mitosis of this type cannot be estimated. An approximation, however, can be made by disregarding any possible variation in mitosis that may arise due to diurnal rhythm. Taking the mitotic rate of the 10 a.m. to 4 p.m. interval (0.30) and multiplying this figure by four, gives a product of 1.20 per cent which is larger than the value (1.11) shown in Table IX. The actual daily mitotic rate lies between these two values. The figure of 1.20 represents a maximum estimate for the daily rate of mitosis.

The daily mitotic rate in the normal alveolar tissue was estimated to be 4.52 (1.13 x 4), (Bertalanffy and Leblond, 1953). The daily production of tumor cells is approximately four times less than that occurring in the alveolar tissue.

The doubling time of the Type 1 tumor is approximately 83 days. This represents the corrected value.

The doubling time based on the colchicine technique is valid if (1) all cells in the population are capable of dividing, (2) there is little or no cell loss, and (3) the rate of division remains constant. Since these assumptions cannot be established with the colchicine technique, previously published doubling times based on this method may require a conservative evaluation (Bertalanffy and Lau, 1962; Bertalanffy, 1963a; 1963b; Bertalanffy and McAskill, 1964). Before such results can be unequivocally accepted confirmatory data from different techniques are required (Mendelsohn, 1962). Therefore the figure of 83 days represents a provisional value for the doubling of the Type 1 alveologenic tumor of the mouse.

4. EXPERIMENT NO. 4

The present results support Rogers (1955) hypothesis that urethane does not have a carcinogenic influence when applied directly to pulmonary tissue.

Rogers exposed fetal mouse lung in vitro to rabbit serum, obtained from urethane injected rabbits. Following transplantation he observed lung tumors in the graft. Simultaneous exposure of lung tissue from the same mice to serum from the same rabbits but taken prior to urethane injection but with urethane added in vitro did not yield any

tumors. Rogers (1955) interpreted these results to suggest that urethane's carcinogenicity is through an intermediate substance or metabolite.

The absence of pulmonary tumors, either alveologenic or bronchogenic in the present experiment may have been due to (1) the condition of the grafted lung, or to (2) the activity of urethane.

It would appear unlikely that grafted lung was incapable of becoming neoplastic in its new environment, since subcutaneous lung transplants develop alveologenic tumors when the hosts are subcutaneously injected with urethane (Shapiro and Kirshbaum, 1951; Heston and Dunn, 1951). Moreover, incorporation of methylcholanthrene into grafts of adult mouse lung (Horning, 1947, 1950, Experiment No. 5) and into fetal mouse lung (Smith, 1950) have resulted in pulmonary tumors. Thus it appears that subcutaneously grafted lung can become neoplastic.

Failure to observe tumors may have been due to an absence of an appropriate carcinogenic stimulus. It may be maintained that the failure to find tumors in the grafted lung was due to an insufficient amount of urethane being incorporated into the graft. Perhaps the lung in this environment requires a larger dose of urethane if it is to respond. Since approximately 1.3 mg. of urethane was placed in the graft as compared to 0.25 mg. each mouse received in

Experiment No. 3, this criticism does not appear to be valid.

The possibility exists that urethane does express itself through an intermediary substance but in the environment of the graft perhaps the necessary oxygen concentration for oxidation reactions are not available.

If an intermediate substance or metabolite formed it remains to be established why this substance once formed had not returned to the graft and stimulated tumor formation. It may be pertinent to mention that attempts to find a metabolic or degradation product of urethane that was effective as the parent substance have been uniformly unsuccessful (Larsen, 1947, 1948; Larsen et al., 1946; Berenblum et al., 1959).

The results of this experiment suggest that urethane does not produce lung tumors when applied directly to grafted lung tissue. As the presence or absence of an intermediary substance, the present evidence is not sufficient to deny its existence yet there is no reason to affirm its presence.

Future experiments with larger doses, other tissues, and sites of transplantation as well as the surgical implantation of urethane crystals directly into the functioning lung of the host may be of value.

5. EXPERIMENT NO. 5

Kreyberg (1962) in his report on the classification of human lung cancer stated, with respect to epidermoid

carcinomas that they are the most common type of humen lung cancer and their rate of increase in the last few decades is closely connected with cigarette smoking.

In the present experiment the most common type of bronchogenic neoplasm was the epidermoid type. It would appear then that Horning's (1947, 1950) method of inducing bronchogenic cancer in mice is satisfactory as a method of yielding an experimental model of lung cancer. The technique has the advantage over intratracheal instillation in that the tumors have a shorter latent period as well as an increased frequency of appearance.

CHAPTER VI

CONCLUSIONS

1. EXPERIMENT NO. 1

(a) I: Ear epidermis

(1) A single painting of benzpyrene in acetone (0.3 per cent) was applied to the ears of 12 mice.

(2) Mitotic rates of the epidermal cells were recorded during the following 24-hour period by the colchicine technique.

(3) Benzpyrene has little effect on the mitotic rates during the first 12 hours after application.

(4) During the remaining 12 hours, benzpyrene blocks the percentage of cells entering mitosis.

(5) Thirty-four per cent fewer cells divided in the 24-hour period following the painting than in unpainted ears.

(b) II: Interscapular epidermis

(1) Sixteen mice were painted once with a benzpyrene in acetone solution on the unshaven interscapular epidermis.

(2) Mitotic rates of the interfollicular epidermal cells for the following 24-hour period were estimated by the colchicine technique.

(3) The hair cycle influenced the mitotic activity in interfollicular interscapular epidermis.

(4) Failure to control the hair cycle made it impossible to separate from a given mitotic rate the percentage of cells dividing due to carcinogenic treatment from the percentage dividing as a result of the growth of the hairs.

(5) It was not possible to determine the influence of a single benzpyrene painting on the mitotic activity of interfollicular interscapular epidermis during the 24-hour period following application.

2. EXPERIMENT NO. 2

(a) I: Interfollicular epidermis

(1) Twice a week for 11 consecutive weeks single paintings of benzpyrene were applied to the unshaven interscapular epidermis of mice.

(2) The mitotic rates of the interfollicular epidermis was estimated at weekly intervals by the colchicine technique.

(3) The mitotic rates followed a pattern consisting of an initial lag period for three weeks, a period of progressive cell increase which reached a peak at seven weeks, a period of inhibition from the seventh to eighth week and finally a terminal stage of mitotic increase to the eleventh week.

(4) The significance of this proliferative

pattern and its relationship to the neoplastic process is not known.

(b) II: Hair follicular epithelium

(1) Multiple paintings of benzpyrene in acetone were administered to the unshaven interscapular epidermis of mice, twice a week for 25 consecutive weeks.

(2) The mitotic rates of hair follicular epithelium was estimated during the histogenesis and regression of a Type 1 keratoacanthoma.

(3) The results suggested that the mitotic activity of the developing, mature and regressing Type 1 keratoacanthoma paralleled the mitotic activity of the hair follicle during its cycle of development, hair production and regression.

(4) Two subtypes of Type 2 keratoacanthoma were recognized on the basis of differences in histology and mitotic rates.

(5) The morphology and mitotic activity of one subtype was similar to that of a Type 1 keratoacanthoma, while the morphology and mitotic rates of the other approached that of an epidermoid carcinoma.

(6) The mitotic rates for a six-hour period of two epidermoid carcinomas were both approximately 16 per cent. The doubling time was estimated to be 1.6 days.

3. EXPERIMENT NO. 3

(1) Of the 36 alveologenic tumors microscopically examined 209 days after a single injection of urethane to 16 mice, one tumor was classified as a papillary adenocarcinoma while the remainder were classed as papillary adenomas.

(2) Two papillary adenomas (Type B) which were morphologically identical to the others had a mitotic rate for a six-hour period of 1.0 per cent.

(3) The remaining 33 papillary adenomas (Type A) had a mitotic rate for a six-hour period that ranged from 0.05 to 0.39 per cent.

(4) A diurnal rhythm was indicated in Type A papillary adenomas.

(5) The daily mitotic rate of Type A tumors was estimated to be 1.20 per cent which is four per cent lower than that of normal alveolar mouse tissue.

(6) The doubling time of Type A adenomas was estimated to be 83 days.

(7) The papillary adenocarcinoma had a six hour mitotic rate six times that of a papillary adenoma (Type A) growing in the same lung.

4. EXPERIMENT NO. 4

(1) Adult mouse lung tissue was removed and in vitro

impregnated with 1.3 mg. of urethane crystals and implanted into the subcutaneous tissue of homologous mice.

(2) No tumors were observed at 77, 105, 212 and 343 days after implantation.

(3) Urethane does not produce lung tumors when applied directly to grafted lung tissue.

5. EXPERIMENT NO. 5

(1) Adult mouse lung tissue was removed and in vitro impregnated with 0.2 mg. of methylcholanthrene and transplanted to the subcutaneous tissue of homologous mice.

(2) At sacrifice, 109 days after implantation, 18 tumors were recovered from 25 mice, one tumor from each mouse.

(3) There were nine epidermoid carcinomas, four fibrosarcomas, four undifferentiated carcinomas, and one adenocarcinoma.

(4) Mitotic rates of the epidermoid carcinomas and of the adenocarcinoma was estimated by the colchicine technique.

(5) The average mitotic rate for a six-hour period of the epidermoid carcinoma was 5.11 per cent. The adenocarcinoma had a mitotic rate for a six hour period of 2.06 per cent.

(6) Horning's (1947; 1950) technique of inducing bronchogenic tumors in mice in a comparatively short time with a relatively high yield was confirmed

BIBLIOGRAPHY

Andervont, H. B. (1937) Pulmonary tumors in mice IV. Lung tumors induced by subcutaneous injection of 1, 2, 5, 6-dibenzanthracene in different media and by its direct contact with lung tissues. Pub. Health Rep. 52:1584-1589.

Andervont, H. B. (1938) Pulmonary tumors in mice. V. Further studies on the influence of heredity upon spontaneous and induced lung tumors in mice. Pub. Health Rep. 53:232-237.

Andervont, H. B. (1939a) Pulmonary tumors in mice. VII. The serial transmission of lung tumors occurring in inbred mice. Pub. Health Rep. 52:1519-1524.

Andervont, H. B. (1939b) Pulmonary tumors in mice. VI. Time of appearance of tumors induced in strain A mice following injection of 1, 2, 5, 6-dibenzanthracene or 20-methylcholanthrene. Pub. Health Rept. 54:1512-1519.

Andervont, H. B., and Shimkin, M. B. (1940) Biologic testing of carcinogens. II. Pulmonary-tumor-induction technique. J. Nat. Cancer Inst. 1:225-239.

Argyris, T. S. (1963) Hair growth cycles and skin neoplasia. NCI Monogr. No. 10:33-43.

Baserga, R., Tyler, S. A., and Kisielski, W. E. (1963) The kinitics of growth of the Ehrlich tumor. Arch. Path. 76:9-13.

Berenblum, I. (1949) The carcinogenic action of 9, 10-dimethyl-1, 2-benzanthracene on the skin and subcutaneous tissues of the mouse, rabbit, rat, and guinea-pig. J. Nat. Cancer Inst. 10:167-174.

Berenblum, I., Ben-Ishai, D., Haran-Ghera N., Lapidot, A., Simon, E., and Trainin, N. (1959) Skin initiating action and lung carcinogenesis by derivatives of urethane (ethyl carbamate) and related compounds. Biochem. Pharm. 2: 168-176.

Bertalanffy, F. C. (1963a) Mitotic rate of spontaneous mammary gland adenocarcinoma in C3H/HeJ mice. Nature 198: 496-497.

Bertalanffy, F. C. (1963b) Mitotic rate of methylcholanthrene induced subcutaneous fibrosarcoma. Naturwissenschaften 50:648-649.

Bertalanffy, F. D. (1964) Respiratory tissue: structure, histophysiology, ctydodynamics. Part I. Review and basic cytomorphology. Int. Rev. Cytol. 16:233-328.

Bertalanffy, F. D., and Chosen Lau C. (1962) Cell renewal. Int. Rev. Cytol. 13:357-366.

Bertalanffy, F. C., and Leblond, C. P. (1953) The continuous renewal of the two types of alveolar cells in the lung of the rat. Anat. Rec. 115:515-541.

Bertalanffy, F. D., and McAskill, C. (1964) Rate of cell division of malignant mouse melanoma B16. J. Nat. Cancer Inst. 32:535-545.

Blacklock, J. W. S. (1957) The production of lung tumors in rats by 3, 4,-benzpyrene, methylcholanthrene, and the condensate from cigarette smoke. Brit. J. Cancer. 11:181-191.

Blacklock, J. W. S., and Burgan, J. G. (1962) The carcinogenic effects of various fractions of cigarette condensate. Brit. J. Cancer. 16:453-459.

Blumenfeld, C. M. (1943) Studies of normal and abnormal mitotic activity of experimental epidermoid carcinomas in mice. Arch. Path. 35:667-673.

Brookes, P., and Lawley, P. D. (1964) Evidence for the binding of polynuclear aromatic hydrocarbons to nucleic acids of mouse skin: relation between carcinogenic power of hydrocarbons and their binding to desoxyribonucleic acid. Nature. 202:781-784.

Brown, C. E. (1963) Carcinoma of the rat lung from intrapleural methylcholanthrene. Arch. Path. 76:347-353.

Bullough, W. S. (1948) Mitotic activity in the adult male mouse, Mus musculus L. The diurnal cycles and their relation to waking and sleeping. Proc. Roy. Soc. B. 135: 212-233.

Bullough, W. S. (1962) The control of mitotic activity in adult mammalian tissues. Biol. Rev. 37:307-342.

Bullough, W. S., and Laurence, E. B. (1960) The control of epidermal mitotic activity in the mouse. Proc. Roy. Soc. B. 151:517-536.

Cameron, I. V. (1964) Is the duration of DNA synthesis in somatic cells of mammals and birds a constant? J. Cell Biol. 20:185-188.

Cameron, I. V., and Greulich, R. C. (1963) Evidence for an essentially constant duration of DNA synthesis in renewing epethelia of the adult mouse. J. Cell Biol. 18:31-40.

Champy, C., and Vasiliu, I. (1923) Beserches sur le cancer experimental du goudron. Essai d'une theorie generals des cancers epethellaux basee sur les faits connus de la biologie des epetheliums. Bull. de l'Assoc. franc. p. l'etude d. cancer 12:111-134.

Chase, H. B., Momtagna, W., Malone, J. D. (1953) Changes in the skin in relation to the hair growth cycle. Anat. Rec. 116:78-81.

Cloudman, A. M. (1941) Spontaneous neoplasms in mice. In: G. H. Snell (ed.); Biology of The Laboratory Mouse, p. 168, Philadelphia, Blakiston Co.

Cooper, Z. K., and Franklin, H. C. (1940) Mitotic rhythm in the epidermis of the mouse. Anat. Rec. 78:1-8.

Cooper, Z. K., and Keller, H. C. (1942) Mitotic frequency in methylcholanthrene epidermal carcinogenesis in mice. J. Nat. Cancer Inst. 2:335-344.

Cowen, P. N. (1947) Some studies on the action of urethane on mice. Brit. J. Cancer. 1:401-405.

Cramer, W., and Stowell, R. E. (1942) The early stages of carcinogenesis by 20-methylcholanthrene in the skin of the mouse. II. Microscopic tissue changes. J. Nat. Cancer Inst. 2:379-402.

Cramer, W., and Stowell, R. E. (1943) Skin carcinogenesis by a single application of 20-methylcholanthrene. Cancer Res. 3:36-42.

Cronkite, E. P. (1959) Discussion of availability time of labelled DNA precursors. In: F. H. Stohlman (ed.); The Kinetics of Cellular Proliferation, p. 117, New York, Grune and Stratton.

Cronkite, E. P., Fleidner, T. M., Killmann, S. A., and Rubini, J. R. (1962) Tritium-labelled thymidine (H^3TDR); its somatic toxicity and its use in the study of growth rates and potentials in normal and malignant tissue of man and animals. In: Tritium in the Physical and Biological Sciences, Vol. 2, p. 807. Vienna, International Atomic Energy Agency.

Croxton, F. E. (1958) Elementary Statistics. New York: Dover Publication, Inc., 276 pp.

Dammert, K. (1961) A histological and cytological study of different methods of skin tumorigenesis in mice. Acta. Path. Microbiol. Scandinav. 53:33-49.

Daoust, R. (1959) Mitotic activity in mouse epidermis during the induction period of papillomas. Acta. Path. Microbiol. Scandinav. 45:159-166.

Della Porta G., Kolb, L., and Shubik, P. (1958) Induction of tracheo-bronchial carcinomas in the Syrian golden hamster. Cancer Res. 18:592-597.

Deringer, M. K. (1962) Response of strain HB/De mice to painting with urethane. J. Nat. Cancer Inst. 29:1107-1121.

Edwards, J. L., Koch, A. L., Yoncis, P., Freese, H. L., Laite, M. B., and Donalson, J. T. (1960) Some characteristics of DNA synthesis and the mitotic cycle in Ehrlich ascites tumor cells. J. Biophys. Biochem. Cytol. 7:273-282.

Eigisti, O. J., and Dustin, P. (1955) Colchine in Agriculture, Medicine, Biology and Chemistry. Ames, Iowa, State College Press.

Evensen, A. (1961) Changes in the synthesis of desoxyribonucleic acid (DNA) and in mitotic count in epidermis of hairless mice after a single application of one per cent 3-methylcholanthrene in benzene. Acta. Path. Microbiol. Scandinav. Suppl. 148:43-52.

Evensen, A., and Iversen, O. H. (1962) Rate of cell proliferation in a mouse squamous cell carcinoma. Nature, 196: 383-384.

Foulds, L. (1951) Experimental study of the course and regulation of tumor growth. Ann. R. Coll. Surg. Engl. 9:93-101.

Friedkin, M., Tilson, D., and Roberts, D. (1956) Studies of desoxyribonucleic acid biosynthesis in embryomic tissues with thymidine -C^{14}. J. Biol. Chem. 220:627-637.

Fry, R. J. M., Lesher, S., and Kohn, H. I. (1961) Estimation of time of generation of living cells. Nature. 191:290-291.

Ghadially, F. N. (1958) Comparative morphological study of kerato-acanthoma of man and similar experimentally produced lesions in rabbit. J. Path. and Bact. 75:441-453.

Ghadially, F. N. (1959) Experimental production of kerato-acanthomas in hamster and mouse. J. Path. and Bact. 77:277-782.

Ghadially, F. N. (1961) The role of the hair follicle in the origin and evolution of some cutaneous neoplasms of man and experimental animals. Cancer. 14:801-816.

Glücksmann, A. (1945) The histogenesis of benzpyrene-induced epidermal tumors in the mouse. Cancer Res. 5:385-400.

Grady, H. G., and Stewart, H. L. (1940) Histogenesis of induced pulmonary tumors in strain A mice. Am. J. Path. 16:417-432.

Haaland, M. (1908) Spontaneous tumors in mice. Imperial Cancer Research Fund (Gr. Brit.) Third Scientific Report 175-261.

Ham, A. W., and Leeson, T. S. (1961) Histology, 4th ed., 942 pp. J. B. Lippincott Co., Philadelphia and Montreal.

Hartwell, J. L. (1951) Survey of compounds which have been tested for carcinogenic activity. United States Government Printing Office, Washington.

Herrold, K. M., and Dunham, L. J. (1962) Induction of carcinoma and papilloma of the tracheobronchial mucosa of the Syrian hamster by intratracheal instillation of benzo(a)pyrene. J. Nat. Cancer Inst. 28:467-491.

Heston, W. E., and Dunn, T. B. (1951) Tumor development in susceptible strain A and resistant strain L lung transplants in LAF_2 hosts. J. Nat. Cancer Inst. 11:1057-1071.

Hill, A. B. (1955) Principles of Medical Statistics, Oxford University Press, 6th ed. New York. 367 pp.

Horning, E. S. (1947) Induction of bronchogenic carcinomas in mice. Lancet, ii, 207-209.

Horning, E. S. (1950) Studies on the induction of lung cancer in mice. Brit. J. Cancer. 4:235-244.

Howard, S., and Pelc, S. R. (1951) Nuclear incorporation of P^{32} as demonstrated by autoradiographs. Exptl. Cell Res. 2:178-186.

Hughes, E. (1949) The induction of transplantable bladder carcinoma in inbred strains of mice. J. Urol. 62:837-841.

Hughes, W. L., Bond, V. P., Brecher, G., Painter, R. B., Quastler, H., and Sherman, H. G. (1958) Cellular proliferation in the mouse as revealed by autoradiography with tritiated thymidine. Proc. Nat. Acad. Sci. 44:467-483.

Iversen, O. H., and Evensen, A. (1962) Experimental skin carcinogenesis in mice. Acta. Path. Microbiol. Scandinav. Suppl. 156:1-184.

Jobling, J. W. (1910) Spontaneous tumors of the mouse. Monographs, Rockefeller Inst. Med. Research, 1:81-119.

Karrer, H. E. (1956) The ultrastructure of mouse lung. J. Biophys. and Biochem. Cytol. 2:241-259.

Karrer, H. E. (1958) The ultrastructure of mouse lung: The alveolar macrophage. J. Biophys. and Biochem. Cytol. 4:693-700.

Kennaway, Sir E. (1955) The identification of a carcinogenic compound in coal-tar. Brit.Med. J. 2:749-752.

Kiljunen, A. (1956) Mitotic activity in normal and malignant epidermal tissue of the rat. Acta. Path. Microbiol. Scandinav. Suppl. 112:1-101.

Kisieleski, W. E., Baserga, R., and Lisco, H. (1961) Tritiated thymidine and the study of tumors. Atompraxis 7:81-85.

Klinken-Rasmussen, L. (1954) Quantitative morphological studies on cyclic changes in the mouse skin with special reference to carcinogenesis. 2: mitotic activity in the epidermis and hair follicles during the first two hair cycles. Acta. Path. Microbiol. Scandinav. 35:523-536.

Kreyberg, L. (1962) Histological lung cancer types. Acta. Path. Microbiol. Scandinav. Suppl. 157:1-92.

Kuschner, M., Laskin, S., Christofano, E., and Nelson, N. (1956) Experimental carinoma of the lung. In: Proc. Third Nat. Cancer Conf. Philadelphia, J. B. Lippincott, pp. 485-495.

Larsen, C. D. (1947) Evaluation of the carcinogenicity of a series of esters of carbamic acid. J. Nat. Cancer Inst. 8:99-101.

Larsen, C. D. (1948) Pulmonary tumor induction with alkylated urethanes. J. Nat. Cancer Inst. 9:35-37.

Larsen, C. D., Rhoads, P. B., and Weed, L. L. (1946) Survey of hypnotics in general with regard to pulmonary tumor induction in mice. J. Nat. Cancer Inst. 7:5-8.

Leblond, C. P., and Bertalanffy, F. D. (1951) Reticulin membranes as the framework of alveolar lung tissue in the albino rat. Canadian Med. Assn. J. 65:263-264.

Leblond, C. P., and Walker, B. E. (1956) Renewal of cell populations. Physiol. Rev. 36:255-276.

Lipkin, M., Sherlock, P., and Bell, B. M. (1962) Generation time of epethelial cells in the human colon. Nature. 195:175-177.

Livingood, L. E. (1896) Tumors in the mouse. Bull. John Hopkins Hosp. 7:177-178.

Low, F. N. (1952) Electron microscopy of the rat lung. Anat. Rec. 113:437-449.

Low, F. N. (1953) The pulmonary alveolar epithelium of laboratory mammals and man. Anat. Rec. 117:241-263.

McCarter, J. A. (1956) The control of certain factors of dosage in epidermal carcinogenesis. J. Nat. Cancer Inst. 17:399-403.

McCarter, J. A. (1959) Tumor production in relation to benzpyrene in skin. Acta. Unio. Internat. Contra. Cancrum 15:178-180.

Mendelsohn, M. L. (1962) Autoradiographic analysis of cell proliferation in spontaneous breast cancer of C3H mouse. III. The growth fraction. J. Nat. Cancer Inst. 28:1015-1029.

Mendelsohn, M. L., Dohan, F. C., Jr., and Moore, H. A., Jr. (1960) Autoradiographic analysis of cell proliferation in spontaneous breast cancer of C3H mouse. I. Typical cell cycle and timing of DNA synthesis. J. Nat. Cancer Inst. 25:477-484.

Mider, G. B., and Morton, J. J. (1939) Skin tumors following single application of methylcholanthrene in C57 brown mice. Am. J. Path. 15:299-302.

Miller, E. C. (1951) Studies on the formation of protein-bound derivatives of 3,4-benzpyrene in the epidermal fraction of mouse skin. Cancer Res. 11:100-108.

Mostofi, F. K., and Larsen, C. D. (1951) The histopathogenesis of pulmonary tumors induced in strain A mice by urethane. J. Nat. Cancer Inst. 11:1187-1221.

Mottram, J. C. (1934) On the correlation between malignancy and the rate of growth of tar warts in mice. Am. J. Cancer. 22:801-830.

Mottram, J. C. (1936) A further consideration of the growth rates of tar warts in mice and their autografts. Am. J. Cancer. 28:115-120.

Niskanen, K. O. (1949) Observations on metaplasia of the bronchial epithelium and its relation to carcinoma of the lung; pathoanatomical and experimental researches. Acta. Path. Microbiol. Scandinav. Suppl. 80:1-80.

Okada, Y., Daidos, S., and Ishiko, S. (1962) Morphological studies of induced pulmonary tumors in mice with special reference to their cytogenesis. Acta. Tuberc. Jap. 11:373-377.

Orr, J. W. (1947) The induction of pulmonary adenomata in mice by urethane. Brit. J. Cancer. 1:311-316.

Orr, J. W. (1963) The role of the stroma in epidermal carcinogenesis. NCI Mcnogr. No. 10, 531-537.

Owen, M., and MacPherson, S. (1963) Cell population kinetics of an osteogenic tissue. II. J. Cell Biol. 19:33-44.

Pan, S. C., and Gardner, W. U. (1948) Induction of malignant tumors by methylcholanthrene in transplanted uterine cornua and cervices of mice. Cancer Res. 8:613-617.

Pelc, S. R. (1963) On the question of renewal of differentiated cells. Exptl. Cell Res. 29:194-198.

Poel, W. E. (1959) Effect of carcinogenic dosage and duration of exposure on skin-tumor induction in mice. J. Nat. Cancer Inst. 22:19-35.

Poel, W. E. (1963) Skin as a test site for the bioassay of carcinogens and carcinogenic precursors NCI Monogr. No. 10:611-631.

Post, J., Huang, C., and Hoffman, J. (1963) The replication time and pattern of the liver cell in the growing rat. J. Cell Biol. 18:1-12.

Potter, V. R. (1957) Metabolic products formed from thymidine. In: F. H. Stohlman (ed.), The Kinetics of Cellular Proliferation, pp. 104, New York: Grune and Statton.

Pullinger, B. D. (1940) The first effects on mouse skin of some polycyclic hydrocarbons. J. Path. and Bact. 50:463-471.

Pyley, L. M. (1961) Experimental induction of the lung cancer in rats by intratracheal introduction of 9,10-dimethyl-1, 2-benzanthracene. Bul. Exper. Biol. and Med. 52:99-102.

Quastler H., and Sherman, F. G. (1959) Cell population kinetics in the intestinal epithelium of the mouse. Exptl. Cell Res. 17:420-438.

Reichard, P., and Estborn, B. (1951) Utilization of desoxyribosides in the synthesis of polynuchleotides. J. Biol. Chem. 188:839-846.

Reller, H. C., and Cooper, Z. K. (1944) Mitotic incidence in the first 48 hours of methylcholanthrene epidermal carcinogenesis. Cancer Res. 4:206-240.

Ritchie, A. C. (1957) Epidermal carcinogenesis in the mouse by intraperitoneally administered urethane followed by repeated applications of croton oil. Brit. J. Cancer 11: 206-211.

Robertson, J. S., Bond, V. P., and Cronkite, E. P. (1959) Resolution and image spread in autoradiographs of tritium-labelled cells. Int. J. Appl. Radiat. 7:172-177.

Rogers, S. (1955) Studies of the mechanism of action of urethane in initiating pulmonary adenomas in mice. I. The indirect nature of its oncogenic influence. J. Nat. Cancer Inst. 15:1675-1683.

Schultze, B., and Oehlert, W. (1960) Autoradiographic investigation of incorporation of H^3 thymidine into cells of the rat and mouse. Science. 131:737-738.

Selbie, F. R., and Thackray, A. C. (1948) Lung adenomas induced by urethane in CBA mice. Brit. J. Cancer. 2:380-385.

Shabad, L. M. (1962) Experimental cancer of the lung. J. Nat. Cancer Inst. 28:1305-1332.

Shapiro, J. R., and Kirschbaum, A. (1951) Intrinsic tissue response to induction of pulmonary tumors. Cancer Res. 11:644-647.

Sherman, F. G., Quastler, H., Wimber, D. R. (1961) Cell population kinetics in the ear epidermis of mice. Exptl. Cell Res. 25:114-119.

Shimkin, M. B. (1955) Pulmonary tumors in experimental animals. Adv. Cancer Res. 5:223-267.

Shimkin, M. B., and Lorenz, E. (1942) Factors influencing the induction of pulmonary tumors in strain A mice by carcinogenic hydrocarbons. J. Nat. Cancer Inst. 2:499-510.

Shimkin, M. B., and Polissar, M. J. (1955) Some qualitative observations on the induction and growth of primary pulmonary tumors in strain A mice receiving urethane. J. Nat. Cancer Inst. 16:75-97.

Shimkin, M. B., and Polissar, M. J. (1958) Growth of pulmonary tumors in mice of strains A and C3H. J. Nat. Cancer Inst. 21:595-610.

Shubik, P., Baserga, R., and Ritchie, A. C. (1953) The life and progression of induced skin tumors in mice. Brit. J. Cancer. 7:342-351.

Shubik, P. H., and Hartwell, J. L. (1957) Survey of compounds which have been tested for carcinogenic activity. Suppl. I. United States Government Printing Office, Washington.

Siegler, R., and Duran-Reynals, M. L. (1962) Observations on the pathogenesis of experimental skin tumors. A study of the mechanism by which papillomas develop. J. Nat. Cancer Inst. 29:653-673.

Slye, M., Holmes, H. F., and Wells, H. G. (1914) The primary spontaneous tumor of the lungs in mice: Studies in the incidence and inheritability of spontaneous tumors in mice. J. Med. Res. 30:417-442.

Smith, W. E. (1949) The tissue transplant technic as a means of testing materials for carcinogenic action. Cancer Res. 9:712-723.

Smith, W. E. (1950) The neoplastic potentialities of mouse embryo tissue. V. The tumors elicited with methylcholanthrene from pulmonary epithelium. J. Exper. Med. 91:87-103.

Stevens, Hooper, C. E. (1961) Use of colchicine for the measurement of mitotic rate in the intestinal epithelium. Am. J. Anat. 108:231-244.

Stevenson, J. L., and von Haam, E. (1963) Induction of pulmonary tumors in C57BL mice using strings impregnated with 20-methylcholanthrene. Acta. Cytol. 7:126-128.

Stewart, H. L. (1959) Pulmonary tumors in mice. In: F. Homburger and W. Fishman (ed.), Physiopathology of Cancer, pp. 18-37, 2nd ed. New York: Hoeber-Harper.

Storey, W. F., and Leblond, C. P. (1951) Measurement of the rate of proliferation of epidermis and associated structures. Ann. N.Y. Acad. Sci. 53:537-545.

Svoboda, D. J. (1962) Ultrastructure of pulmonary adenomas in mice. Cancer Res. 22:1197-1201.

Telford, I. R., and Jeffery, G. A. (1953) A method for the evaluation of tumor growth. Texas Rep. Biol. and Med. 11: 570-572.

Tyzzer, E. E. (1907) A series of twenty spontaneous tumors in mice, with the accompanying pathological changes and the results of the inoculation of certain of these tumors into normal mice. J. Med. Res. 17:155-197.

Tyzzer, E. E. (1909) A series of spontaneous tumors in mice with observations on the influence of heredity on the frequency of their occurrence. J. Med. Res. 21:479-518.

Voutilainen, A. (1953) Uber die 24-Stunden-rhythmik der mitosenfrequenz in malignen Tumoren. Acta. Path. Microbiol. Scandinav. Suppl. 99.

Wells, H. G., Slye, M., and Holmes, H. F. (1941) The occurrence and pathology of spontaneous carcinoma of the lung in mice. Cancer Res. 1:259-261.