CYTOKINETICS OF MOUSE EPIDERMIS DURING BENZOPYRENE CARCINOGENESIS

by

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ABSTRACT

Utilizing an improved method of analyzing labelled mitoses curves obtained by tritiated thymidine administration, a variety of growth parameters, tC, tG_1, tS, tG_2 and tM, the mitotic index, and the fraction of cells in DNA synthesis were determined in normal, preneoplastic and neoplastic epithelium of mouse skin.

The fraction of cells in the S phase (nS), the durations of the S, and G_2 phases varied relative to the stages of the hair growth cycle in untreated interfollicular epidermis (IFE). For instance, in Telogen about three times more epidermal cells were synthesizing DNA than during Anagen VI.

The initial reaction of a confined area of epidermis to ten sequential daily applications of benzopyrene in benzene was a decrease of the mitotic duration (tM). Subsequently, the depressed tM returned to the normal control values where it remained until the epidermal tumors developed. At the same stage, the IFE of non-tumor-bearing animals, although likewise treated with benzopyrene, also exhibited a shorter tM. The generation time (tC) of the tumor cells was drastically reduced, primarily due to a shortened duration of the G_1 phase.

The duration of the DNA synthesis phase (tS) of the tumor cells was similar to that of normal and preneoplastic IFE. However, the tS of the tumor cells exhibited less variability than the cells of the normal and preneoplastic IFE populations.

The regressing keratoacanthoma displayed a particularly brief G_2 phase. Apart from this tumor, the tG_2 phases of the other tumor types were within the control range.

The tM of the malignant epidermal tumors proved to be the most

variable phase encountered.

Benign tumors displayed a higher mitotic index than either the normal or the preneoplastic IFE. The fraction of cells in the S phase (nS) did not vary significantly in the different tumor cell populations; but this fraction was higher in malignancy than in the normal and preneoplastic IFE. However, the hyperplastic IFE 40 days after benzopyrene treatment displayed nS between that of other preneoplastic and tumor cell populations.

DEDICATION

To my wife, Joan, without whose patience and constant encouragement this thesis would not have been attempted, much less completed.

"When it is not in our power to determine what is true, we ought to act according to what is most probable."

Descartes.

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INTRODUCTION

I. PROBLEMS

1. Normal Epidermis

Cytokinetics of normal interfollicular epidermis (IFE) relative to the major stages of the hair growth cycle: a) Telogen; and b) Anagen VI.

The purpose of this experiment was to determine the range of growth activity by which a cell is affected relative to the hair growth cycle.

2. Carcinogen Treated Epidermis

Cytokinetics of IFE treated with benzopyrene at regular intervals after the initiation of carcinogenesis at intervals of a) 20 days; b) 40 days; c) 60 days; d) 80 days; e) 100 days; f) 120 days.

The purpose of this experiment was to determine the effect of a continuous application of carcinogen to mouse IFE during Telogen. The histological appearance of the preneoplastic IFE was likewise studied. The experiment was extended until most of the surviving animals had developed epidermal tumors.

3. Induced Tumors

The cytokinetics of the tumors induced by the action of the carcinogen: a) benign tumors; and b) malignant tumors.

The purpose of this experiment was to ascertain which compartments of the cell cycle became altered while tumors developed.

II. DEFINITIONS

The following is a list of definitions and/or abbreviations of terms used in the text.

Benign tumor - a tumor which remains quite localized, does not invade

surrounding tissues, causes no harm except by virtue of its position or accidental complication (Willis, 1960).

BP - benzopyrene, 1,2-benzopyrene or 3,4-benzopyrene.

Carcinogen - any substance that induces malignancies (Webster's 1966). It may be a physical, chemical or biological agent.

Epidermis - the outermost and nonvascular epithelial layer of the skin (Dorland, 1965). In this report, it constitutes specifically the interfollicular epidermis and pilosebaceous apparatus (Vide infra).

DMBA - 9:10-dimethyl-1:2-benzanthracene.

EKA - early keratoacanthoma.

IFE - interfollicular epidermis consisting of the epidermal regions between hair follicles (Fig. 1).

KA - keratoacanthoma, a type of benign epidermal tumor derived from the hair follicle walls. Subdivided into types, such as, KA1 is the keratoacanthoma type 1.

Labelling index - the percentage of cells synthesizing DNA at any time. Determined 1 hour after a single pulse label of tritiated thymidine. See also nS.

Malignant tumor - a tumor that invades neighbouring tissue, spreads by metastasis, and unless extirpated at an early stage, inevitably proves fatal (Willis, 1960).

MCA - 3-Methylcholanthrene.

Mitotic activity - the degree of cell divisions in a cell population (Wallace, 1964).

Mitotic duration - the time required for a cell to pass through the morphological phase of mitosis, that is, from prophase through telophase (Leblond and Walker, 1956).

Mitotic index - the number of mitotic figures (all stages of mitosis) occuring in a cell population at any instant of time; it is usually determined by scoring 1000 cells.

Mitotic rate - the percentage of cells undergoing cell division per unit time interval, as during a 6-hour period.

MKA - mature keratoacanthoma.

N - fraction of cells in the proliferating compartment.*

nC - number of proliferating cells.*

nS - fraction of cells synthesizing DNA.*

Pilosebaceous apparatus - comprising the hair follicle and its associated sebaceous glands (Dorland, 1965).

RKA - regressing keratoacanthoma.

SCC - squamous cell carcinoma.

tC - generation time.*

tG_1 - duration of presynthetic (post mitotic) phase.*

tG_2 - duration of post synthetic (premitotic) phase.*

tM - duration of mitosis.*

tS - Duration of DNA synthesis.*

Tumor - any abnormal mass of tissue, the growth rate of which often exceeds and is uncoordinated with that of the normal tissue of origin, and persists in the same excessive manner after cessation of the stimuli which provoked the change (Willis, 1960).

* Quastler, 1963

III. AIMS OF THIS STUDY

Despite the circumstance that the epidermis was the first tissue in which chemical carcinogenesis was demonstrated, and this tissue has

been studied extensively, controversy still surrounds not only the carcinogenic mechanism and its effect on the normal growth controlling mechanisms, but also the normal growth controlling mechanism itself. The variables which influences this homeostatic mechanism resulting in the cellular steady-state conditions of the epidermis are numerous, and this thesis attempts to measure one normal variable in particular, namely the hair growth cycle as related to conditions of preneoplasia and neoplasia.

Because of the interactions between renewing cells and a chemical carcinogen or its active metabolites, a cell population homeostatic mechanism becomes altered to allow an increase in the number of cells in a population. It is believed that during this period of homeostatic imbalance the malignant cell arises and flourishes. A disturbance in this mechanism can be observed by determining several parameters of cell growth, before, during and after carcinogen treatment, since the parameters of growth which were studied were a reflection of the cybernetic interaction of cells.

Before man can understand the neoplastic phenomenon, and before he can determine rational therapy to the problem of neoplasia, the normal growth controlling mechanisms of homeostasis must be explored and ascertained. Having understood how homeostasis is maintained, one may attempt to comprehend the altered patterns of growth which are called "cancer". Previously, little work was concerned with the range of proliferative activity of cells becoming tumors. In many investigations the normal state is poorly controlled, and exhibits a wide variance. Many neoplasms are stated to exhibit a spectrum of mitotic behaviours, implying that the mitotic activity varies so widely that mean values determined are of little significance. This thesis attempts to ascertain normal parameters more accurately and determine many parameters of growth with an expression

of variance. These expressions of variance are in many cases very crude but they are a rough method of statistically comparing possible differences.

CHAPTER II

REVIEW OF THE LITERATURE

1. Microscopic Anatomy of Mouse Skin

a) Interfollicular Epidermis (IFE) The IFE of the dorsal skin of the mouse is composed usually of a single, but nor more than two layers of cells, overlain by a relatively abruptly appearing, narrow band of keratin. Most of the cells are in contact with, and adhere to the dermo-epidermal junction, forming a slightly undulating line. The cytoplasm is basophilic, and the cells contain one round or oval nucleus per cell. Mitotic figures appear only in scattered cells and were encountered only in the basal layer if more than one layer was present (Setala et al., 1959). Glucksmann (1945) was the first to describe different cell stages in the epidermis of the mouse; he and other authors have reported the frequency of the cell types as:

	basal cells	differentiating cells	degenerating cells	mitotic cells
Glucksmann (1945)	71.1%	25.4%	3.48%	0.06%
Setala et al. (1959)	71.0%	25.0%	4.00%	0.06%
Niskanen (1962)	76.4%	19.0%	4.46%	0.14%
Iversen and Evensen (1962)	60-65%	35-40%		

Iversen and Evensen (1962) observed that the average number of cell layers was approximately 1.6 in hairless mice. Niskanen (1962) determined the distribution of cell types in RA female mice. The longitudinal axis of the nuclei of the basal cells in female mice of that strain was ascertained to be perpendicular to the basement membrane. In contrast, differentiating cells had their long axis parellel to the cutaneous surface. The histology of the skin of the RA mice and Swiss CF, female mice was essentially similar (Nyyosson, 1966). Iversen and Bjerknes (1963) expressed the opinion that the 'stratum germinativum' of normal mouse epidermis was soley confined to the basal layer. These authors stated

further that the relative amount of cell types in the IFE of hairless mice was composed of about 60% basal cells and about 40% differentiating cells. The concensus of recent investigations (Elgjo, 1969) has been that in stratified epithelia new cells become formed in the basal layers, and each new cell retained the potentiality of reentering division. As new cells are formed in the basal layer, other cells are slowly displaced into the intermediate or differentiating layers. Because both daughter cells remained in the basal layer at least temporarily after mitosis the plane of cell division must normally be parallel to the dermo-epidermal junction.

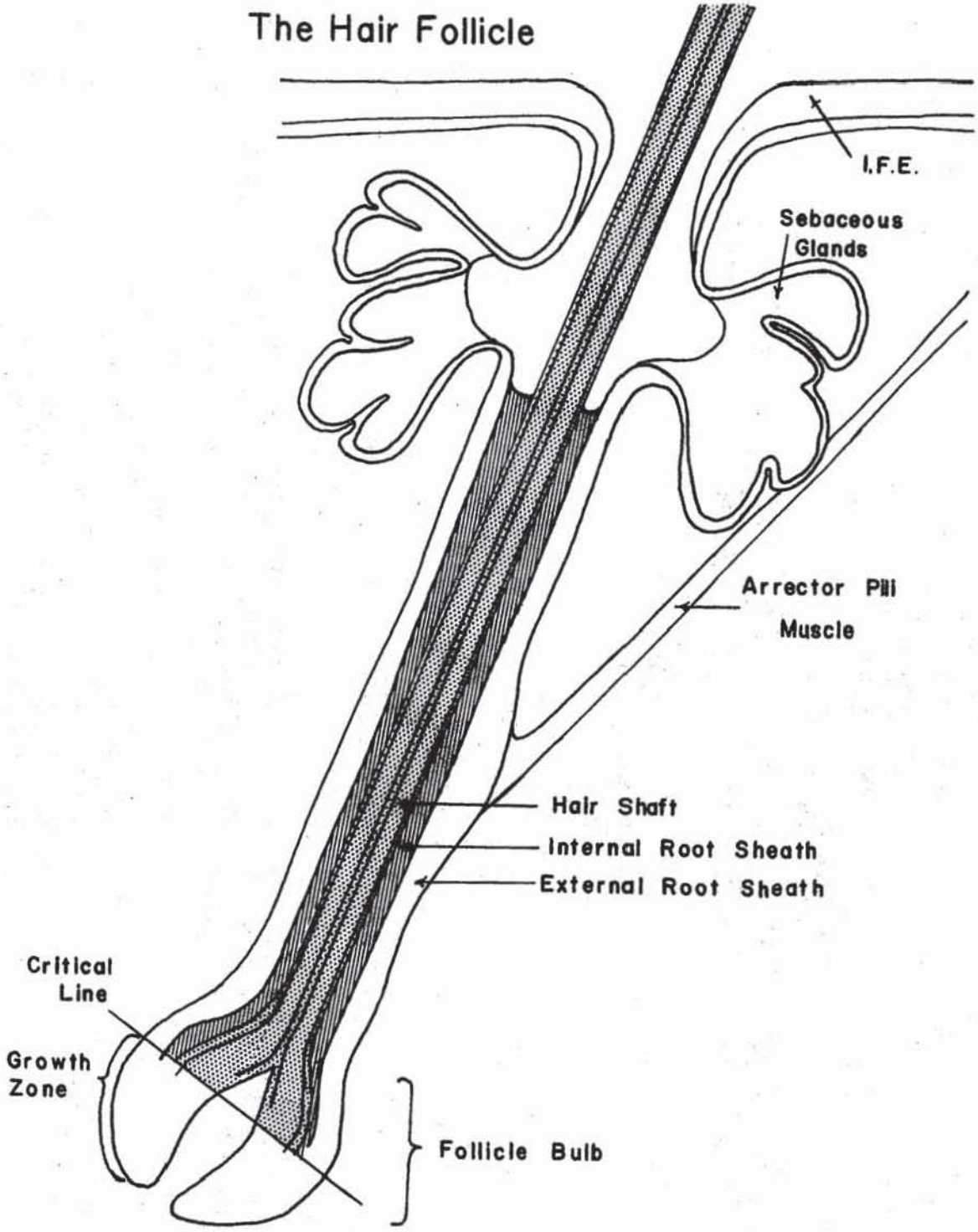
The epidermal appendages of hairless mice consisted of scattered sebaceous glands associated with rudiments of hair follicles, sweat glands, cyst-like formations beneath the epidermal surface, and abundant thin walled cysts in the deeper layers of the skin (Skjaeggestad, 1964).

The ultrastructure of normal haired mice was shown to remain unaffected of the hair growth cycle (Setala et al.,1960). The basal cells of the IFE were attached to the dermoepidermal junction by special attachment "devices" (hemi-desmosomes). Contiguous cell boundaries were intimately associated and followed each other in an intricate, folded curtain-like fashion. Between the basal cells of mouse IFE, intercellular spaces or bridges were absent. However, they occurred between the more superficial differentiating cells.

Mouse IFE, a relatively simple structure with easy accessability for treatment, represents an excellent system for the study of cellular response to topical application of carcinogens and other irritants.

b) Pilosbaceous apparatus: The pilosebaceous apparatus comprises a hair shaft, a follicle, a follicle bulb, a sebaceous gland, and

Figure 1


The Hair Follicle
I.F.E.
Sebaceous Glands
Arrector Pili Muscle
Hair Shaft
Internal Root Sheath
External Root Sheath
Critical Line
Growth Zone
Follicle Bulb

an arrector pili muscle (Fig. 1).

A follicle may contain one growing hair, which extends from the follicle bulb to protrude above the surface of the skin, and one or more resting hair shafts. The resting hair shafts (or hair clubs) eventually fall out or epilate.

The arrector pili muscles are attached to the side of the follicle so as to form an obtuse angle to the surface. They extend to the uppermost layer of the dermis (Ham, 1969). The arrector pili muscles are not involved during epidermal carcinogenesis, and are thus not mentioned further in this report.

The hair follicle is composed of a thin sleeve of epithelium continous with the surface epidermis. The follicles slant into the dermis, and to some extent also below the level of the dermis into the panniculus adiposus; the latter is a layer of adipose tissue of variable thickness.

The hair shaft is a long slender filament of keratinized cells lying within the hair follicle and protruding above the skin. The inner root sheath surrounds the shaft from the matrix to the level of the sebaceous gland. The follicle is composed of (1) the outer root sheath, which is continuous with, but thinner than the IFE; and (2) the outer capsule, a specialized portion part of the dermis (Montagna and Ellis, 1958; Montagna and van Scott, 1958; Montagna, 1962) (Fig. 1).

The follicle bulb is an enlargement of the follicle at its lowermost or deepest portion. That tissue situated below an imaginary line (the critical line, Fig. 1) which passes through the widest part of the hair follicle, except the papilla, is called the matrix; below this level the cells proliferate most rapidly. The dermal papilla, is

composed of a richly vascularized connective tissue; it is constricted at its base and pointed toward the apex. The ultrastructure of mouse hair follicle in Telogen was described by Roth (1965) and in Anagen by Roth and Helwig (1964a, 1964b).

The growing mouse hair follicle matrix is supported by a distinct basement membrane; however the type of cells into which the matrix cells will differentiate eventually cannot be predicted. By contrast, six concentric tubes of cells can be distinguished in the upper segment of the follicle. Although the cells of both areas resemble each other, the matri cells display somewhat less cytoplasm.

Sebaceous glands are holocrine glands composed of two morphologically distinct cells in different stages of differentiation. The sebaceous gland is externally surrounded by a reticulin basement membrane upon which rest the "basal cells". These basal cells proliferate actively by mitosis. They are continuous with the wall of the hair follicle. Within the central cavity, lined by basal cells, are the "sebaceous cells", which after fatty transformation, become the secretion material, sebum (Bertalanffy, 1957).

c) Dermis: The dermis consists largely of dense irregular collagenous fibres, and dermal papillae are absent (except in the nipple region of the mammary gland, Snell, 1941). Below the dermis lies a thin stratum of adipose tissue, which separates the dermis from the panniculus carnosus, the layer of skeletal muscle. The sub-epithelial tissue is composed of dense bundles of collagen fibres, a small number of fibroblasts, and capillaries (Niskanen, 1962).

2. Cell Population Kinetics

The epidermis contains a labile population of cells (Bizzozero,

1894) where mitoses are regularly observed. The horny layer, the product of cell differentiation, is continuously shed from the surface. This keratinous layer of the epidermis is the result of complex cellular interactions involving energy utilizing processes, (Hayashi, 1959; Mercer, 1961).

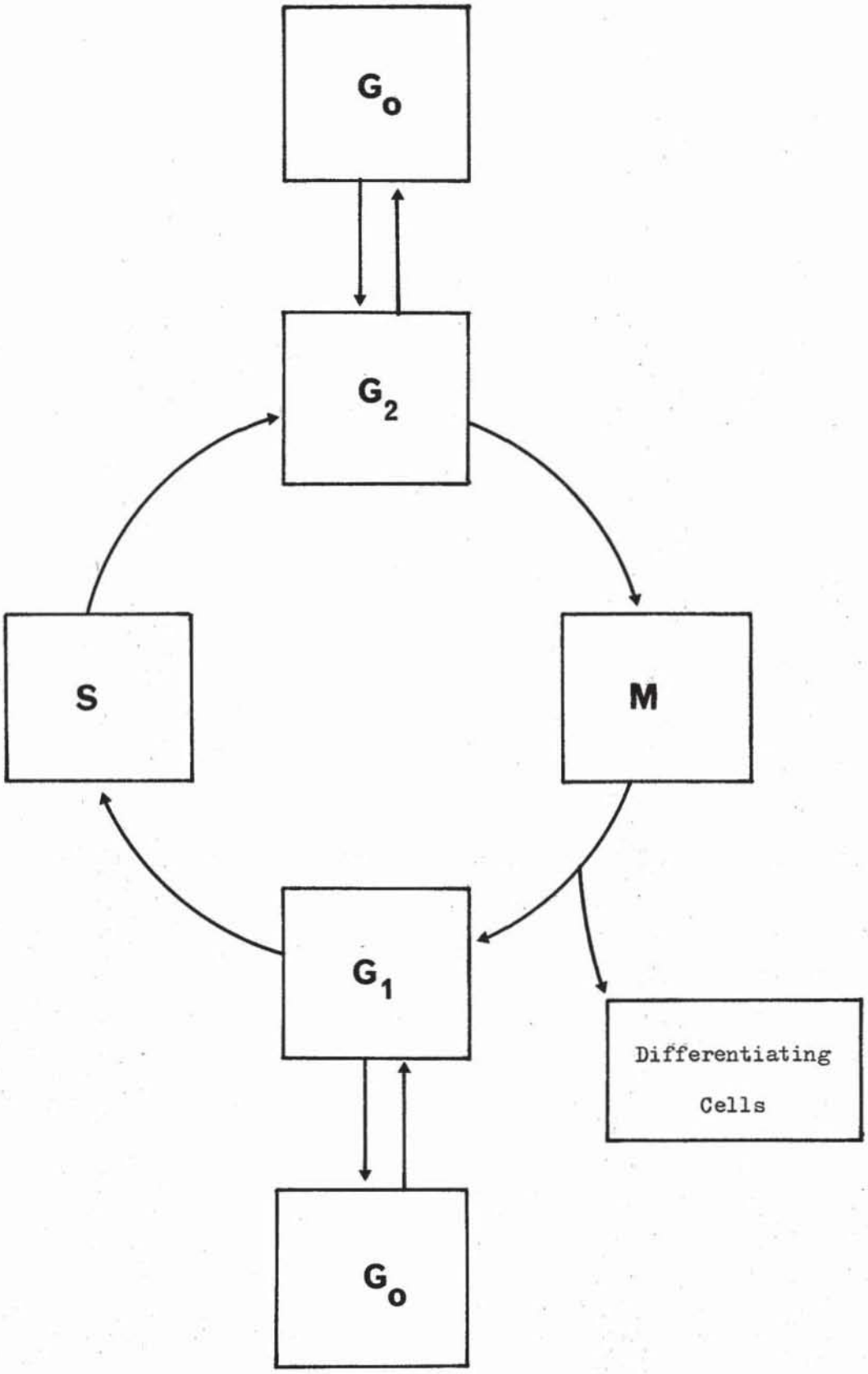
Growth of the epidermis can be measured either as the volume of hair produced or by its mitotic activity. Mitotic activity refers to the magnitude of cell proliferation in a cell population and can be expressed as; (1) the percentage of cell divisions present at any instant, that is, the mitotic index; (2) the percentage of cell divisions occuring during a certain period of time, that is, the mitotic rate; (3) the percentage of cells becoming labelled by a suitable radioactive tracer, that is, the radiographic or labelling index.

Before discussing methods of growth measurement, the life history or cell cycle is discussed. Two cell fractions are found in renewing cell populations, the proliferation fraction and the differentiating fraction. Cells in the differentiating fraction are responsible for the formation of the end-products typical of that particular tissue. In mouse epidermis the end-product is the keratinized part of the epidermis. These differentiated, keratinized cells are continually sloughed off or desquamated from the surface (Fig. 2). Cells in the proliferating fraction are engaged in replacing the cells thus lost.

a) <u>The Cell Cycle</u>: The cell cycle was subdivided into four phases (Howard and Pelc, 1953) (Fig. 2), namely, the post mitotic gap (G_1), the phase of DNA synthesis (S), the premitotic gap (G_2) and mitosis (M). G_1 or the post mitotic gap, is that period between the end of mitosis and the initiation of the synthesis of DNA. Many RNA and non-

Figure 2

Cell Cycle of Growing Interfollicular Epidermis



histone proteins are mostly synthesized during G_1. Most of the cells destined to differentiate leave the cell cycle at the beginning of this phase. The differentiating cells do not return to the cell cycle; they are destined to die without dividing again. Other cells enter what some investigators call a long G_1 phase or G_o phase (Patt and Quastler, 1963; Quastler, 1963; Latjtha, 1967). Cells in G_o can be stimulated to rapidly re-enter the cell cycle and proliferate soon after that stimulation. In some cases this group of cells which may re-enter the cell cycle leave from G_2 (Frankfurt, 1967; Gelfant, 1962), or remain arrested in it. "The flow of continously dividing cells from G_1 to S seems to require the synthesis of RNA and proteins, including special enzymes, like thymidine kinase, but.....the trigger that activates this macromolecular synthesis" is unknown (Baserga, 1968). G_1 is the most variable phase of the cell cycle. In rapidly proliferating populations it may be as brief as one hour. In slowly proliferating cells G_1 may last several days. Some authors consider non-proliferating cells, such as the hepatocytes and nerve cells, to be also in G_1. In such instances, maximum duration of G_1 of these cells is almost equal to their life span. Others group them into the G_o category of cell populations.

S is the phase when DNA is synthesised. In order for a cell to enter this phase, adequate pools of nucleotides, DNA polymerase(s), and DNA activation are necessary to initiate the S phase; yet, these factors alone are insufficient for the regulation of S phase (Prescott, 1964a, 1964b).

In the production of DNA, the double-stranded DNA molecule unwinds and each primer DNA acts as a template to replicate new DNA from four components, adenine, guanine, cytosine, and thymine. The

reaction is catalyzed by DNase and results in an exact replica of the molecule.

The technique of radioautography allows direct visualization of the cells involved in DNA synthesis. In the majority of mammalian cells, the S phase ranges between 6 to 8 hours. One principal exception seems to be mouse ear epidermis. During the S phase, as well as G_1 and G_2, also RNA is synthesized in the nucleus (Baserga, 1966).

G_2, or premitotic gap, is the time between the completion of DNA synthesis and the beginning of mitosis. Formation of RNA essential for mitosis was completed by the end of S or very early in G_2. However, proteins required for mitosis are synthesized up until the end of G_2 (Kishimoto and Lieberman, 1964). The duration of G_2 in most mammalian cell populations ranged from 1 1/2 to 2 1/2 hours (Baserga, 1966). Mitosis is the process of actual division of the cell, subdivided into its four phases of Prophase, Metaphase, Anaphase and Telophase (Bloom and Fawcett, 1968, Ham, 1969). During mitosis, RNA synthesis ceases and the rate of protein synthesis decreases (Baserga, 1968). Ribosomes of mitotic cells are less active than in interphase (Salb and Marcus, 1965). Some cells, such as intestinal crypt cells or those of hair matrix in Anagen VI apparently divide incessently; consequently the majority of these cells actually never cease passing through the cell cycle. Other cells divide very rarely but can be stimulated to return into the cell cycle, for example liver cells after partial hepatectomy. These quiescent cells are considered by some authors as being in a long G_1. Patt and Quastler (1963), as others, postulated that these cells were in a G_o period. Most other categories of cells leave the cell cycle and differentiate to a point beyond which they are unable to alter

their metabolic machinery to revert to mitosis. This differentiating fraction usually leaves the cycle after mitosis but according to some authors, occasionally also after G_2 (Frankfurt, 1967).

Thus, Gelfant (1961) postulated that cells in the IFE of mouse ears sometimes left the cell cycle in G_2. Such withdrawal of cells from the proliferating fraction may have been brought about by the low temperature in which the cells in these experiments were growing. 92% of cells which were stimulated into mitosis by wounding mouse ears were immediately derived from the G_2 phase. These cells had been in G_2 (or a G_2 derived G_o compartment) for at least 48 hours prior to wounding (Gelfant, 1966). G_1 cells were also stimulated by wounding to move into S phase but only 24 hours later (Gelfant, 1963a). Thus, there was a 24 hour delay in DNA synthesis and subsequent mitosis when ears were wounded in vivo. Such delay was not apparent when the ears were wounded in vitro (Gelfant, 1966). By raising substantially the environmental temperature, Storey and Leblond (1951)observed an increased number of cells in mitosis in rat plantar epidermis. Also, Gelfant (1966) demonstrated in mouse ear epidermis, that heat shock could stimulate many more cells that were resting in G_2 (or G_2 derived G_o cells) to enter cell division. That author suggested further that there were at least three subpopulations of G_2, namely those that could be stimulated to divide in vitro by the addition of a) glucose, b) Na^+ ions or c) K^+ ions to the culture. He also postulated that those subpopulations posessed specific and different requirements for mitosis.

The biochemistry of the cell cycle was excellently reviewed by Baserga (1968). Many substances alluded to in that review were capable of blocking the entrance of cells into various compartments of the cell cycle.

Several methods are available for estimating the fraction of cells within a specific stage. A complete review of this cohort of literature available is not warranted here. Watanabe and Okada (1967) discussed the principal methods applied to cell populations in tissue culture. Many such procedures were applied to in vivo experiments or are suitable for both in vivo and in vitro studies.

The cell cycle generation time (tC) is the time interval between any stage of the cycle in the parent cell to the attainment of the identical point of the cycle by one or both of the daughter cells.

b) Mitotic Index: The mitotic index of a proliferating cell population is that number of dividing cells present at any one instant among 1000 cells. Measurement of the mitotic index was employed for calculating the mitotic activity of human cell populations, because cytostatic drugs (such as Colchicine) cannot readily be administered (Scheving, 1959). Another application of the mitotic index was to estimate the mitotic duration. Disadvantages of the mitotic index are that it is affected by the mitotic duration. Moreover, large numbers of cells have to be enumerated to achieve statistically sifnificant observations (Reller and Cooper, 1944). Finally, the mitotic index is not readily ascertained accurately when the mitotic activity of a cell population is low.

c) Colchicine Technique: The chemical colchicine is plant alkaloid extracted from the seeds, flowers and corms of the saffron plant, Colchicum autumnale; it is an alkaloid crystalline substance. Stevens-Hooper (1961) noted that the prophase incidence was identical in tissues whether or not the animals were treated with colchicine. She ascertained that when colchicine was applied in proper dosage, it arrested all cells in metaphase without stimulating or depressing the normally

occuring mitotic activity. Colchicine allows these "arrested colchicine metaphases" to accumulate for specified periods of time, thus facilitating ascertainment of mitotic rates and cellular growth (Eigsti and Dustin, 1955).

The optimum dose of Colchicine, administered subcutaneously, was determined to be for rats, 0.1 mgm. per 100 gram body weight, and for mice, 0.2 mgm. per 100 gram body weight. The most expedient period of colchicine action was reported to be four to six hours (Bertalanffy and Leblond, 1953). Stevens-Hooper (1961) ascertained that there was a fifteen minute delay before arrested epidermal cells were actually observed arrested in metaphase. Chivers (1967) confirmed this observation, true also of the hair matrix cells. However, the first arrested metaphase in the IFE were noticeable only 25 minutes after administration of colchicine. This phenomenon was possibly related to differences in blood supply to the two regions of skin.

Taylor (1965), employing radioactive tritiated colchicine, observed that within 15 minutes of its administration, colchicine had penetrated the cells and became equilibrated with the extracellular colchicine concentration in the tissue fluid. The same author demonstrated that colchicine, after having been bound to particular cytoplasmic components of the interphase cells, became gradually lost also from the cells after it became removed from the surrounding medium.

The particular advantages of the colchicine technique is that it furnishes data on percentages of cells dividing during a certain period, and a single figure for the turnover time of the cell population (Bertalanffy, 1964).

d) Mitotic Duration: is the time required for a cell to enter

morphological prophase until it separates into two daughter cells. Because the actual early stages of cell division are difficult to recognize the precise estimate of this interval is not always easy. Leblond and Stevens (1948) demonstrated that the mitotic duration (M) can be determined according to the relation

$$tM = \frac{x\ M.\ I.}{M.R.\ \text{for } x \text{ hours}} \qquad (1)$$

where M.I. is the mitotic index, M.R. is the mitotic rate for a given number of hours and x is the number of hours over which the M.R. was estimated. This estimate may be very accurate when the number of arrested metaphases are used to estimate MI and M.R. But, it is less accurate when the total number of mitotic figures is employed.

Another means to estimate of the mitotic duration is according to the formula (Quastler and Sherman, 1959; Bennington, 1969)

$$tM = \frac{tC\ nS}{tS} \qquad (2)$$

where tC = generation time, nS is the percentage of cells synthesizing DNA and tS is the duration of S phase.

A third mode of estimating the duration of M is from the labelled mitosis curve. At 50% y intercept are two roots of this curve, the time from zero to the lower root represents a fraction of M plus G_2. It is usually expressed as $1/2\ M + G_2$ (Sherman et al., 1961). However, whether or not the fraction is in fact 1/2 M is unknown.

An <u>in vitro</u> method of determining the mitotic duration was proposed for asynchronous cell populations (Stanners and Till, 1960) by the formula

$$tM = \frac{M.I.\ tC}{100 \ln 2} \qquad (3)$$

The duration of mitosis had previously been held to be fairly

constant (Mazia, 1961). More recent studies have revealed, however, that the mitotic duration varied between tissues. And this variance did not only occur between cell populations, but in mouse epidermis the mitotic duration was demonstrated to vary from hour to hour relative to the changes of the diurnal mitotic cycle (Bullough and Laurence, 1964a). Bullough and Laurence (1966a) concluded that the lower the adrenalin of chalone concentration was in body tissues, the larger was the number of mitoses that occured and also the more rapidly each mitotic division was completed.

e) Tritiated Thymidine Technique (H^3-T): Thymidine, a specific precursor of thymine in DNA synthesis, when tagged with tritium (H^3-T), becomes incorporated into the DNA molecules of those cells replicating their DNA, for about an hour after administration. Once incorporated into nuclei, it is diluted further only during subsequent cell divisions (Hughes et al., 1958). Blenkinsopp (1967) ascertained that injected H^3-T did not affect the rate of entry of cells into the S phase of the tongue or esophageal epithelium of C_3H male mice. Thus, when H^3-T is available, the nuclei of all cells synthesising DNA during that period become labelled, and can be visualized by radioautography. The radiographic (or labelling) index is determined by calculating the percentage of labelled cells within a cell population (Messier and Leblond, 1960).

The advantages of this technique are that it requires a relatively small number of animals, the time necessary to evaluate the observations is brief, and the cells become labelled permanently, an expedient feature especially in the study of cellular migration (Bertalanffy, 1964).

The tritium which labels thymidine is an isotope of hydrogen with a mass 3, and a half-life of 12.3 years. It decays by the emission of a particle with an average energy of only 5.7 kev. The maximum energy is about 18 kev. (Robertson and Hughes, 1959). Ninety percent of the β particles of tritium are absorbed by 1.2 μ of a medium of unit density (Fitzgeraldn and Vinijchaikul, 1959). In a photographic emulsion of density 3.5, 99% of the radiation becomes absorbed within 0.8 μ (Taylor, 1960).

By employing a pulse label of H^3-T, Quastler and Sherman (1959) were able to label a cohort of cells which were synthesizing DNA. By killing animals at one hour intervals after H^3-T administration, they were able to observe these cells as they passed through mitosis. For the first few hours, labelled mitotic figures were not observed; subsequently labelled mitotic figures began to appear and their number became augmented to eventually approach 100%. Several hours later the percentage of labelled mitotic figures declined and eventually approached zero. The time between administration of H^3-T and the point at which the ascending limb was at 50%, represented the overall duration that a cell spent between S phase and mitosis. The period between the two 50% levels was the duration of S (tS). The percentage of cells which were labelled at one hour represented that fraction of cells that were in S phase (nS) when H^3-T was available. The generation time (tC) could be determined from the formula

$$tC = \frac{tS \times N}{nS} \qquad (4)$$

where tS = DNA synthesis time,

N = % of cells growing.

nS = % of labelled cells.

However, Johnson et al. (1960) suggested that this procedure did not provide an expedient estimate of cell populations with a long generation time (or with cell populations in which all the cells were not proliferating). They proposed alternatively for calculation of the generation time the formula

$$tC = \frac{tS\ (\ln 2)}{\text{labelled cells/total cells}} \qquad (5)$$

where ln 2 = 0.69315.

Provided all cells are proliferating, the calculated tC represents the true tC. But if all of the cells are not in the proliferating compartment then the calculated tC will be greater than the true tC. Equation (5) was based on the assumption that the distribution of cells throughout the cell cycle was not uniform but that the population was asynchronous. This equation also presupposes that the growth fraction (N) was equal to ln 2.

If the generation time (tC) was known then the percentage of proliferating cells could be ascertained from formula (4)

$$N = \frac{tC\ nS}{tS} \qquad (6)$$

as suggested by Quastler and Sherman (1959) and Bennington (1969).

An additional estimate of the mitotic duration (M) becomes feasible with that data.

$$tM = \frac{MI \times tC}{N} \qquad (7)$$

When M is known 1/2 M and G_2 may be calculated. The duration of G_1 is estimated by subtraction

$$tG_1 = tC - (tG_2 + tS + tM) \qquad (8)$$

When the mitotic duration is thus estimated, a second calculation

of the fraction of cells in the proliferation compartment becomes feasible.

$$N = \frac{MI \times tC}{tM} \qquad (9)$$

The duration of S phase in normal tissues has been ascertained in many instances to range from 7 to 30 hours. Bullough and Laurence (1966) suggested that there was a widespread belief that 7 hours was the mean time required for DNA synthesis in most mammalian tissues.

For the estimation of the percentage labelled mitosis curve, Thrasher (1966) stated that a minimum of 100 labelled and unlabelled mitoses should be scored per animal. He suggested that in tissues with a slow rate of proliferation, at least 20 mitoses be enumerated. In regard to the present investigation, Stevens (1968; personal communication) proposed that the number of mitoses in a constant base population be enumerated. Inasmuch the base population in ascertaining the mitotic rate, mitotic index and labelling index was 2000 cells, the same number was adopted also as the base population when scoring labelled and unlabelled mitosis.

Thrasher (1966) pointed out that all animals should be killed at the same time of the day, preferably when the diurnal rhythm of mitosis was at a peak of activity. This procedure is particularly noteworthy, but unfortunately became known to the author only at a later date during the experiments. For sake of uniformity in execution of the experiments this method was thus not adopted in the present investigations.

The percentage of labelled interphase nuclei at 30-90 minutes after H^3-T administration was a reliable indicator of the relative size of the progenitor population in S phase. Thus

$$tC = \frac{tS \times 100}{nS} \qquad (10) \text{ Thrasher (1966).}$$

2. Biological Effects of Tritiated Thymidine

After tritiated thymidine (H^3-T) is injected into an animal the fate of the compound may be two-fold. The H^3-T may be catabolized either to tritiated water (H^3OH) and other compounds. Cronkite et al. (1959) determined that 25 to 50% of H^3-T injected into human patients was recoverable as H^3OH. He also recovered from urine 5 - 10% of the injected H^3-T as β-aminoisobuturic acid and other non-volatile tritiated compounds. Steel (1962) demonstrated that about 40% of the H^3-T injected into rats was catabolized in the liver to H^3OH, and a few percent non-volatile activity was recovered from the excreta. One hour after injection of H^3-T, the concentration of H^3OH remained almost constant. This indicated that no further H^3-T became catabolized.

The amount of H^3OH present in a particular tissue after H^3-T injection is of significance only when total radioactivity is measured, as by liquid scintillation counting.

The second route that H^3-T may take is to be incorporated into the nucleus of cells replicating DNA. Following administration, the H^3-T is rapidly taken up by cells in the S phase (DNA synthesizing phase) and transferred to the nucleus. Here an equilibrium becomes established between H^3-T and H^3-T-phosphates (Crathorn and Shooter, 1960). Various authors have shown that H^3-T administered by an intraperitoneal injection entered the nuclear DNA particularly rapidly. Potter (1959) determined that the activity of C^{14} - thymidine reached a maximum in rat spleen and thymus as soon as 2 minutes after an intraperitoneal injection. He suggested that the conversion of thymidine to thymidine phosphate was

a rate-limiting reaction and that conversion of H^3-T-phosphates to DNA was rapid. Rubini et al. (1960) reported that incorporation of H^3-T occurred as soon as one minute after an injection into patients and was nearly completed after 10 minutes. Nygaard and Potter (1959) demonstrated that the maximal incorporation of H^3-T into DNA, called the incorporation value, was attained within one hour. This value depended on several factors, one of them was the rate of DNA synthesis; yet it was not paralleled by the incorporation value.

There exists conclusive evidence that DNA of the cell nucleus is a very stable compound, and when labelled with H^3-T the label remains in the DNA of the cells throughout their life span or else until subsequent division. Leblond et al.,(1959) ascertained in various tissues that the autoradiographic reaction overlying cell nuclei was at the same level of intensity 6 months after an injection of H^3-T into 3 day old mice as the level of activity had been at 3 days after the label was administered.

Labelling of proliferating bone marrow cells remained in the cells for their life span, and became diluted only by successive mitosis (Rubini et al., 1962; Bond et al., 1962). The stableness of H^3- DNA was true both on a molecular level as on a chromosomal level during the first 4 - 5 divisions after the labelling (Cronkite et al., 1962).

The mechanism whereby DNA is broken down in vivo is unknown. Conceivably, DNA is hydrolysed by DNase, and converted to nucleotides and nucleosides by a variety of phosphatases. The thymidine itself could be further broken down to β-aminoisobutyric acid (Davidson, 1960).

When labelled cells of the thymus were allowed to degenerate in the presence of macrophages, the latter phagocyosed the H^3- DNA . Even

after these macrophages, the latter phagocyosed the H^3- DNA. Even after these macrophages divided, they had not incorporated any labelled material into their own DNA. Although the same was true for mouse embryo cells, it was not the case for HeLa cells (Mims, 1962). Skjaeggstad (1964) concluded from such observations that "Re-utilization of H^3- DNA and its breakdown products is small or non-existent". Any possible re-utilization of H^3- DNA that might occur is not of significance in regard to the present study. Skjaeggstad (1964) determined further that within the range of his experiments (mice aging from 10 to 22 weeks) the activity of H^3- DNA in epidermal tissues resulting from an injection of 50 μc H^3-T was independent of sex, age, or body weight.

When DNA is labelled with β-emitting isotopes, such as H^3-T, cellular changes occur occasionally which could be interpreted to be radiation effects. Thus, Painter et al., (1968) were able to kill tissue culture cells with H^3-T. Further, chromosomal aberrations were induced by H^3-T in plant root tips (Wimber, 1959; Natarajan, 1961).

Garder and Devik (1963) demonstrated that cellular death by irradiation from the H^3 label in H^3-T occurred in mice administered a large dose of 200 μc H^3-T. They assumed that even 20 μc H^3-T might cause some cell death. Such effect, if it occurs, would be minimal in the epidermis exposed to H^3-T for a period of 12 hours. Mendelsohn (1960) was unable to observe any effect on growth survival in auto-transplanted mammary gland tumors in mice even after 50 to 100 μc H^3-T had been injected 4 weeks previously.

Although radiation effects have been described as being produced by H^3-T, such effects depend upon the dosage levels and vary with the type of cell population. The epidermis seems to be affected very little,

if at all, by such factors.

3. Hair Growth Cycle

Hair growth affected the thickness of the entire skin, the condition of the epidermis, the activity of the sebaceous glands, the activity of the hair follicles, and the reaction of the skin to various environmental factors (Chase et al., 1953; Chase, 1958 and 1965).

a) Growth: Hair growth proceeded, in different animal species, in various patterns of cycles. There was a seasonal growth of hair in the dog, culminating in a hair molt, (Wolbach, 1951). An interrelationship between phases of hair follicles was not observed in man; rather, the cycles within a given follicle proceeded independently from those of neighbouring follicles (Ryder, 1965).

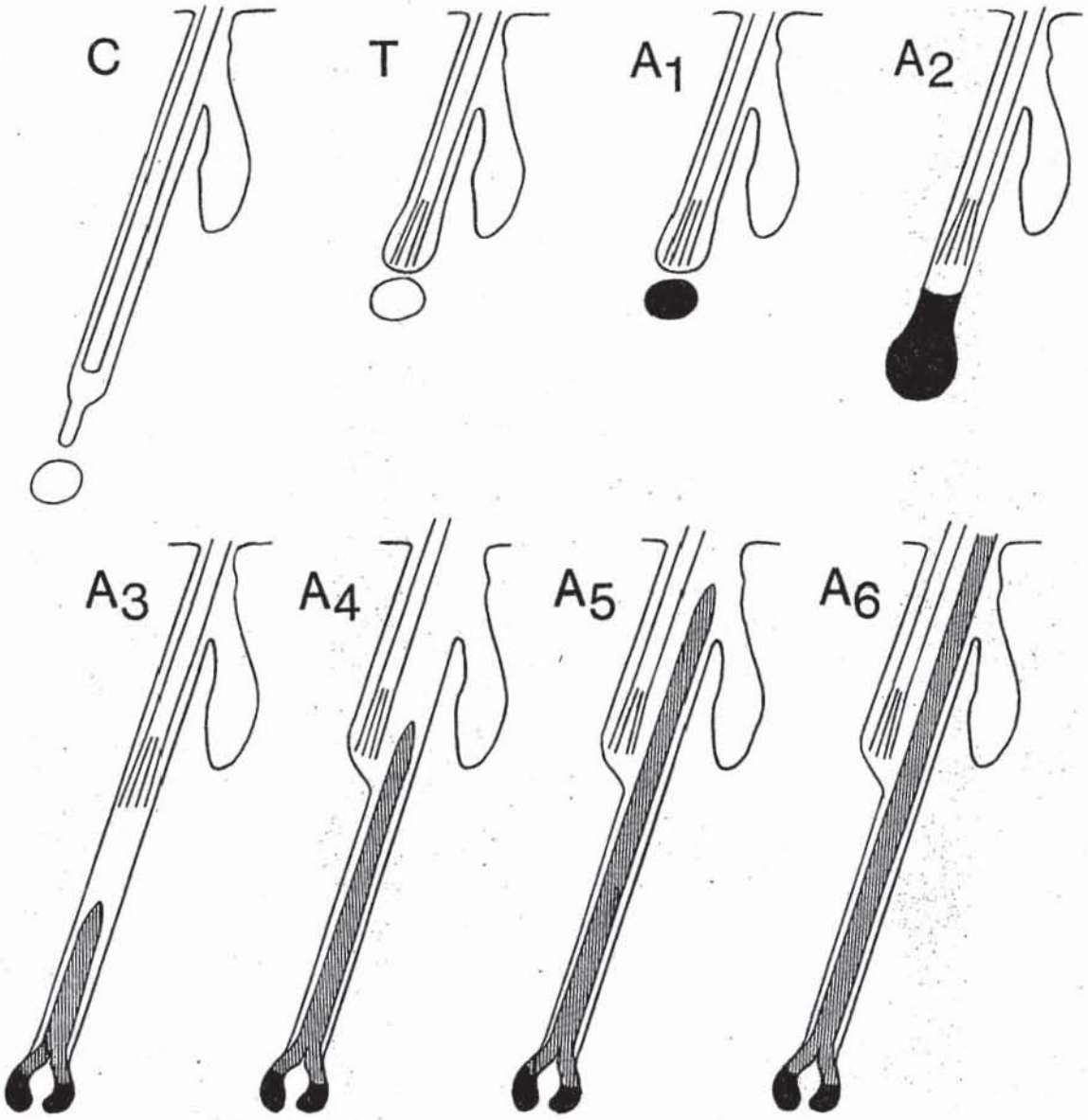
In contrast, the activity of hair growth in rats and mice spread as a wave in a characteristic course and pattern over the various body regions (Borum, 1954). Adjacent areas tended to be in the same phase of the hair growth cycle. This feature rendered these animal species most suitable for studies of hair growth.

The hair growth cycle passes through the following phases in mouse epidermis.

Telogen (T), (Fig. 3) or resting stage, lasted for about ten days. During this time the epidermis was only one to two cell layers thick. The length of the follicle was about one half to one third the height of the growing follicle. Moreover, the Telogen follicle was devoid of a follicle bulb. The deepest extension of the hair shaft contained a brush-like attachment within a clavate base. Below the follicle occured a clump of cells, the hair germ. During the increase of follicular length, that is, between Telogen and Anagen, the dermis and epidermis

Figure 3

Normal Hair Cycle



thickened, concurrent with a size decrease of the sebaceous glands.

Anagen, the growing stages, was subdivided into six phases, Anagen I to VI (Chase et al., 1951, Chase, 1954).

Anagen (A_1) resembled morphologically Telogen, apart from a burst of mitotic activity in the hair germ. Such areas of high mitotic activity are indicated in Fig. 3 by solid black shading.

During Anagen II (A_2) the external root sheath of the follicle became formed. The growth region extended over the entire dermal papilla as it increased in follicular length. The internal root sheath became formed subsequently.

Anagen III (A_3) commenced when the matrix of the bulb was discernible, and the newly formed hair shaft within the follicle was apparent. The tip of the hair shaft attained the level just below that of the bulk of the sebaceous glands.

After the bulb reached its greatest depth below the skin's surface (approximately 500μ) Anagen IV (A_4) commenced. Cellular proliferation (to supply cells for subsequent keratinization) was restricted to the matrix. The upper tip of the hair shaft now attained the level of the sebaceous glands.

In Anagen V (A_5), when the hair bulb gained its greatest diameter, the tip of the hair shaft had grown past the level of the sebaceous glands but did not yet protrude above the skin's surface.

Once the hair shaft extended from the surface of the skin, the final anagen stage, A_6, ensued. The actively growing mouse hair shaft, in Anagen VI (A_6), lengthened at a rate of about one millimeter per day (Montagna and Ellis, 1958).

Catagen (C), the transistional stage, commenced approximately fifteen days after the onset of Anagen I. Cell division ceased, the

follicle became reduced to about 1/4 of its original length. As the hair shaft moved towards the surface, a brush-like device formed at its proximal end and attached to the developing clavate base (Straile et al., 1961).

Montagna (1962) considered Catagen to be a degenerative process. Kligman (1959) described a differentiation of cells during that stage. However, Straile (1962) noted that Catagen was merely one step in an orderly sequence of events in the differentiation of the hair bulb cells which remained after mitosis had ceased (Ryder, 1965).

The relative durations of the stages of the mouse hair growth cycles, after its artificial initiation by hair plucking (of 6 - 7 weeks old mice) were determined by Chivers (1967). It was also observed that the overall duration of the hair cycle varied in mice of the same sex and strain between successive hair cycles. Plucking a somewhat younger mouse initiated a hair cycle which was slightly shorter (present study), than that of an older animal.

Chase et al., (1953) observed that the thickness of the corium in C57Bl/6CH mice was 300 μ in Anagen, and became reduced to 200 μ in Telogen. The layer of adipose tissue in the panniculus adipose likewise decreased simultaneously from 800 μ to 400 μ, respectively. The enhanced thickness of the corium during follicular growth was partially explained by an increase of the capillary network just prior to Anagen (Durward and Rudall, 1949).

Moffat (1968) demonstrated that the thickness of the IFE changed after the hair was plucked. Its thickness decreased from the second day after plucking to become thinner by the eleventh to fifteenth days.

The basal lamina of the ahir follicle did not remain unchanged during the cycle (Montagna, 1962). During Catagen it became altered

being thin and inconspicuous at first to becoming hypertrophied. The hyaline (glassy membrane) appeared as a thick wrinkled sac (Ellis and Moretti, 1959). The ultrastructure of the hyaline membrane of Anagen follicles consisted of an inner basal lamina and two layers of orthogonally arranged collagen fibers (Parakkal, 1969a, Rogers, 1957). During Catagen the basal lamina underwent extensive pleating (Parakkal, 1969b).

b) Cytokinetics: Male mice, up to eight weeks of age, exhibited a mitotic index of the IFE ranging from 0.5 to 2.0 mitoses per 1000 epidermal cells (Klinken-Rasmussen, 1954a, 1954b). The mitotic index of normal rat IFE was reported to be 1.89 ± 0.20 mitoses per 1000 cells (Kiljunen, 1956). Hopsu and Harkonen (1960) determined the mouse IFE mitotic index to be larger in epidermis with actively growing follicles than in skin with inactive follicles. Setala et al., (1960) likewise demonstrated variation of mitotic activity relative to the hair cycle. Eight hours after administration of tritiated thymidine to mice, the radiographic index of the IFE was about 2% (Messier and Leblond, 1960). Braun-Falco (1964) reported that the first artificially induced hair cycle of albino mice affected the mitotic activity of the IFE. The same author determined the number of mitosis per cm. length of epidermis, four hours after a colcemid injection. The number of epidermal mitoses was larger during Anagen than in Catagen and Telogen IFE.

The mitotic rate of Swiss R(SWR) mouse ear IFE, measured during 6-hour intervals of colchicine action (10 a.m. to 4 p.m.), was ascertained to be 1.56 ± 0.42%; in the interscapular IFE the 6-hour mitotic rat was 3.79 ± 2.79% (Wallace, 1964). This study did not take into account any possible effect of the hair cycle on the mitotic rate. The six hour mitotic rates, of abdominal IFE in growing, adult and senile rats ranged from 0.89 ± 0.06% to 1.6 ± 0.12% (Bertalanffy et al., 1965). The

renewal time of human IFE was estimated by radioautography to be approximately 13 days (Weinstein and van Scott, 1965).

The 6-hour mitotic rates of the IFE estimated by the colchicine technique, were observed to fluctuate with the stages of the hair cycle. The mitotic rate of supralumbar IFE of the male SWR mice was lower during the resting stages (Catagen and Telogen) [0.74 ± 0.34%] and became higher during the growing phases (Anagen) [1.17 ± 0.47%] (Chivers, 1967). The renewal times calculated from these data thus ranged between 25 and 34 days, depending upon the stage of the hair cycle.

Applying simultaneous injection of H^3-T and colchicine, Blenkinsopp (1968) determined that in stratified squamous epithelium of C3H/He 8 week old mice the S phase was 8.4 hours in abdominal, and 34.5 hours in ear epidermis. The generation time of progenator cells was estimated to be 156 hours in abdominal and 532 hours in ear IFE. The 24-hour mitotic rate was calculated to be 10.5% to 11% (abdominal IFE) and 3.08% to 3.54% (ear IFE).

The renewal or turnover time of a cell population is that time required for cells to pass from the germinal layer to the surface hence they shed (Mercer, 1961). The renewal time of the Malpighian layer of mouse ear IFE was 28 days, whereas it was 100 days in human abdominal epidermis and 13 days in the human forearm (Storey and Leblond, 1951). Mercer (1961) cited Platt (1960) as having determined the renewal time of guinea pig epidermis to be 82 days.

According to Defendi and Manson (1963) most mammalian cells in vitro exhibited the following approximate mean parameters: tC = 18 hours; tS = 7 hours; tG_2 = 4 hours.

The following parameters were estimated by Iversen (1964) for

the IFE of (hr/hr) hairless mice: tC = 5 days; MI at 10 a.m. = 0.57 ± 0.3%; nS at 10 a.m. = 2.90 ± 0.15%; tM at 10 a.m. = 5 hours; one hour MR = 0.91% of basal cells; tS at 10 a.m. = 5 hours and the average rate of DNA synthesis was 0.57% of all nuclei per hour. Two hours after a pulse label of H^3-T, 0.4% of the basal cells of the IFE of adult mice were labelled (Edwards and Klein, 1961). Pilgrim (1964) ascertained that the tS of normal haired mouse IFE ranged from 6.2 to 7.1 hours, and of rat IFE from 4.8 to 8.0 hours. Pilgrim et al. (1963) reported that the 24 hour mitotic rate of the abdominal epidermis of rats was 7.4%. Sherman et al. (1961) determined the duration of the S phase in mouse ear epidermis to be from 24.5 to 34.5 hours; tG_2 was 6.5 hours, tG_1 was 22 days and tC was 24 days. The 24-hour mitotic rate of the IFE of the rat abdominal epidermis was ascertained to be 6.6% (Bertalanffy and Lau, 1962), and more recently to range from 4.9% to 8.5% (Bertalanffy et al., 1965).

In the ear epidermis and that of body skin in general of the mouse, the average cell cycle time (tC) was 3 - 5 days, with tG_1 being 2 - 5 days, tG_2 approximately 4 hours and tM approximately 3 hours (Gelfant, 1966). Less than 0.1% of the cells in the population were in mitosis and nS = 1.7% at any instant (Gelfant, 1962, 1963a). Wounding of hair plucking stimulated some of the remaining 98% "dormant" cells to pass through the cell cycle, and all cells divided rapidly. The peak of mitosis occured only 48 hours after wounding as the epidermis attempted the wound closure. A delayed response occurred also after hair plucking. Thus, Gelfant (1963a) determined that 10% of all cells that were stimulated into cell division by wounding 48 hours previously were derived from the G_2 population. These G_2 cells had thus been in G_o for

48 hours before entering division.

The growth fraction (N) in normal human epidermis was N = 5%, the turnover time 13 - 18 days (Epstein and Maibach, 1965). Dormer et al. (1964) estimated that tC in mouse epidermis was approximately 150 hours. The generation time of human epidermis had been demonstrated to be likewise 150 hours (Bresciani, 1968).

Pronounced diurnal cycles of cell proliferation existed in the IFE of mice. The maximal epidermal proliferation by mitosis proceeded during the resting hours of the animal, while minimal cell proliferation occurred during muscular activity (Bullough, 1948a, 1948b). A similar rhythm was reported in man (Fisher, 1967). Strenuous excercise of humans (continuous badminton playing for several hours) had the effect of all but abolishing mitotic activity in the IFE.

Pilgrim et al., (1963) ascertained that there were diurnal variations also of the labelling index, while the grain counts themselves remained unchanged after H^3-T labelling. They noted further that the time, during which the mitotic index was at a maximum, was equal to the time period of maximal labelling of H^3-T. They concluded that the interval of the S phase was constant throughout the day, whereas the number of cells entering the S phase from G_1 fluctuated with the time of day.

Bullough (1963) reported the duration of mitosis in the IFE of mouse ears (in vivo and in vitro) to range from 1.5 to 5.3 hours.

The mitotic index of mouse hair follicles exhibited fluctuations paralleling the hair growth cycle (Klinken-Rasmussen, 1954). During the inactive phase, the follicles of rat epidermis displayed a mitotic index of 1.63 ± 0.65 mitoses per 1000 cells; in contrast, the mitotic index of actively growing follicles was 9.98 ± 0.65 (Kijunen, 1956). Bullough and Laurence (1958) reported that the mitotic activity of the fully grown

follicle was confined to the matrix of the bulb. In Anagen I, it was restricted to the area below the brush-like attachment, and during Anagen II and III all new hair follicular cells became formed. The mitotic activity of the fully grown follicle (Anagen IV to VI) was similar to that of the IFE (see Fig. 3).

Similarly, the rates of cell division of the walls of the follicles exhibited fluctuations with the hair cycle. A low mitotic rate was observed during Telogen (1.32 ± 0.52%). It became augmented during the stages of Anagen in the following manner: A_1, 2.64 ± 0.21%; A_2, 3.72 ± 1.40%; A_3, 2.54 ± 0.93%; A_4, 2.69 ± 1.61%; A_5, 4.26 ± 2.08%; A_6, 2.70 ± 1.47%. During Telogen the mitotic rate of the follicular walls was lowest, merely 0.97 ± 0.20% (Chivers, 1967).

c) Epilation effects: Epilation is "the removal of hair by the roots" (Dorland, 1965). When the hair was plucked during late Anagen stages, the duration of Anagen became shortened; however, the following Telogen phase remained unaffected. However, when the hair was plucked during Telogen, the duration of the Telogen phase became lengthened. Epilation during Telogen resulted in a delay of onset of the next hair cycle. Although the effect of hair plucking during Catagen has not been studied, epilation in early Anagen did not exert any effect on the duration of the subsequent stage (Johnson and Ebling, 1964; Johnson, 1965). To achieve optimal results, the smallest area required to be plucked has to contain at least 1000 follicles (Chase and Eaton, 1959). Following hair plucking, Tabachnik et al., (1966) reported a slight epidermal thickening but without evidence of inflamation of tissue necrosis.

After comparing the effects of hair clipping, epilation by barium sulphate, and by epilation after a single application of DMBA, Borum (1958) demonstrated that the "general histology of the artificially induced hair growth cycle was indistinguishable from that of the normal hair cycle".

d) Relation between the hair cycle and tumor production: Significantly more tumors were induced by single or multiple exposures to a carcinogen if the chemical was applied during Telogen rather than during Anagen (Andreasen and Englebreth-Holm, 1953; Borum, 1954; Berenblum et al., 1958; Hieger, 1961). Whiteley (1960) demonstrated that tumors induced by carcinogen application in Anagen were superficial and bud-shaped, while tumors induced in Telogen were deeper. These tumors were produced in rabbits by multiple paintings with DMBA. Prutkin and Gerstner (1966), using similar methods and animal species, supported further the latter observations.

Thus the keratoacanthoma (KA) can be readily induced by a potent carcinogen, if the latter is applied during Telogen. The resulting number of tumors is then greater than when the carcinogen was applied during the other stages of the hair cycle. The KA might be induced more readily in Telogen because the proliferation of cells is slower during that stage, and also the desquamation rate of cells is likely slower, thus resulting in a slower elimination of carcinogen from the cells to be initiated. An objection to this view is the circumstance that hairless mice likewise develop KA's. Stevanovic (1965) demonstrated in hairless mice that these tumors originated from atrophic hair follicles present in the skin as keratinous cysts.

4. Epidermal Neoplasms in the Mouse

a) Induction: A cause and effect relationship of cancer was first demonstrated in epidermal tissues by Sir Percival Pott in 1775, when he implicated that scrotal cancer in chimney sweeps was caused by chimney soot. Yamagiva and Ichikawa (1918) induced experimentally the first skin cancers in the ears of rabbits in 1914 by repeated applications of coal tar.

An extensive resumé of the history of chemical carcinogenesis, including a discussion of the tobacco problem, was published by Shimkin and Triolo, (1969).

The topical application of a chemical carcinogen by means of a small camel's hair brush has been used most widely since 1918 as skin tumor induction techniques; yet, it constitutes one of the poorer methods as it introduces many sources of error. Several noteworthy suggestions for the control of some of the variables arising during application of the carcinogen were proposed by McCarter (1956) and McCarter et al., (1956). They suggested that the carcinogen be administered by a calibrated needle pipette, and that the area exposed to the carcinogen be carefully controlled. A suggestion which may or may not be valid is to limit the time intervals during which the carcinogen remains on the skin. Simpson and Cramer (1943, 1945) demonstrated that methylcholanthrene (MCA) was present in the sebum of sebaceous glands, and in the keratin layers of mouse epidermis, as early as two minutes after a single topical application. Iversen and Evensen (1962) introduced a procedure for controlling the size of the area exposed to the carcinogen. They employed a pair of forceps to hold a fold of skin. A metal ring with an opening of approximately 0.8 $cm.^2$ was then gently pressed against the double flap of skin

resting on a rubber sponge pillow. Skjaeggestad (1964) modified this forceps to fit a rectangular lumen measuring approximately 3 $cm.^2$. Both studies applied the carcinogen within the confined lumen by pipette.

A single intragastric instillation of MCA, urethan or DMBA, without further treatment, yielded irregular epidermal hyperplasia, particularly in the area of the follicular ostium. This was composed of elongated and compactly arranged, sticky basal-type cells arranged in several layers. Not only were the differentiating cells unevenly layered but they also exhibited variable sizes. Alopecia, sebaceous gland atrophy, and slightly increased mitotic activity were evident. On the other hand, Tween 40, a tumor enhancing agent, produced only a regular arranged hyperplasia in the IFE, and the pilosebaceous apparatus remained preserved (Niskanen, 1962).

Page (1938) investigated the effect of one and several applications of BP and MCA dissolved in benzene, as of benzene itself. He ascertained that there occurred two peaks of nucleolar and nuclear size variations after carcinogen application. The first peak was within the first week of the first application, while the second peak coincided with the time the lesions were first diagnosed to be malignant. MCA produced slightly severer effects than BP. Application of benzene alone caused the nucleoli to become slightly smaller.

After fifteen weeks of biweekly applications of 9:10 dimenthl-1:2 - benzanthracene (DMBA) to the interscapular skin of female mice, Shubik et al., (1953) produced skin tumors in 100% of the surviving animals.

Controlled doses of the carcinogen is a very significant factor because higher doses may induce severe inflammatory reactions and can be lethal to many cells. After applying an exceptionally high dose of

DMBA (0.75 mg.) in acetone, Bond and Orr (1969) described the reaction of the skin of female mice to the carcinogen. Within four days the superficial layers of the epidermis, the distal parts of the hair follicles, as well as the sebaceous glands were either necrotic or completely destroyed. Epilation occurred in some of the mice by the sixth day, and in all the treated animals the hair was entirely epilated by the eleventh day. On the eighth day after the painting, epidermal regeneration began. Hair follicles were differentiated de novo. Four weeks after a single administration of DMBA, promoted by biweekly application of 0.5% croton oil in acetone, tumors that were induced arose from the superficial epidermal areas without involving any part of the follicle itself. The reaction of the entire body of the mice to this severe treatment was widespread. It was thus noted that the ovaries were slightly smaller after treatment, and upon histological examination the oocytes proved to be destroyed. The circumstance that the hair follicle did not play any role in the development of these tumors was partly because the upper portions of the follicles were destroyed by the treatment, and the regenerated IFE, which gave rise to tumors, was itself derived from the lower part of the hair follicles. Iversen and Evensen (1964) induced skin tumors in 80% of hairless mice by mutiple applications of MCA. After fourteen weeks of biweekly paintings of SWR mice with MCA, Ozaki (1964) demonstrated that 80% of the survivors bore skin tumors.

Another method of producing epidermal malignancy was by a combination of a carcinogen (one application or a series of applications within a short interval), followed by promotion with multiple applications of a co-carcinogen. As the co-carcinogen effect is to hasten the

formation of a tumor, it can either be capable or incapable of inducing malignancy by itself. Some common co-carcinogens are Croton oil (Berenblum and Haran-Ghera, 1957), oil offSweet Orange (Meek, 1963), and urethan (Deringer, 1965).

A single application of a carcinogen may be sufficient to incite epidermal malignancy. Law (1941) reported the formation of epidermal tumors in 3 of 4 mice, eight months after a single painting of DMBA. Reller and Cooper (1944) were able to induce tumors in 100% of the surviving experimental mice thirteen weeks after a single painting of the ears with MCA. Two months after a single epidermal painting of 0.05 ml. of a 0.5% solution of DMBA in benzene, Borum (1954b) produced tumors in 96% of the female, and in 81% of the male mice. A critical dose level was determined at which a single application of a carcinogen becomes fully effective. Thus, a single dose of 200 µgm. DMBA in acetone was shown to be the optimally effective amount (Terrecini et al., 1960).

McCarter (1966), as others, indicated that the number of tumors induced per mouse was a linear function of the area of skin treated, and of the logarithm of the amount of carcinogen entering the skin area within a given time. That author suggested that the fitting of dose and effect to a logarithmic function was insignificant theoretically. Ball et al., (1964) ascertained that benign tumors were distributed among mice in accordance with Poissons distribution, that is, the appearance of a tumor was a relatively rare event. McCarter (1966) suggested that this may be evidence for a focal origin of cancer. It also could be argued that the treated region of IFE cells itself was transformed but only focal areas developed tumors because of different micro-or even ultra-micro environments.

The fraction of cells incorporating tritiated thymidine (nS) in the hair follicle was lower after an application of DMBA to mouse skin (McCarter and Quaster, 1962a); at the same time tS of epidermal cells was prolonged (McCarter and Quastler, 1962b). The reduction of nS did not imply that the cells were transformed, as some tissues had a shorter nS but did not produce tumors (Jensen et al., 1963).

McCarter (1966) cited Ball, Smith and McCarter, as having prepared a stable complex of BP with DNA which could not be broken in the ultracentrifuge; it required native DNA for its formation.

Sinclair and McCarter (1964) ascertained that after DMBA was applied to mouse skin, the synthesis of RNA was decreased for at least the following 24 hours. The fraction of cells in the S phase (nS), after a single application of 5% croton oil in mineral oil to the ears of mice, was observed to rise to a peak of activity within 24 hours, and then slowly declined by 72 hours (Shinozuka and Ritchie, 1967). Evenson (1962) reported similar observations.

A single subcutaneous injection of urethane in gelatin produced chromosome abnormalities in mice of different strains and ages (Wakonig-Vaartaja, 1964). Forbes (1965a, 1965b) demonstrated that a single painting of 1% mineral oil produced dermal, blue nevus-like spots in haired, hairless and rhino mice.

In addition to the chemical carcinogens, various physical and biological agents have been implicated to induce malignancies. Among physical agents which have demonstratable carcinogenicity are ultraviolet radiation (Winkelmann et al., 1963), X-irradiation (Glucksmann, 1958) and mechanical stress (Selye et al., 1962).

Although virus-like bodies were not encountered on examination

of MCA induced squamous cell carcinomas, Dmochowski et al. (1967) ascertained that a virus was involved in the formation of ocular squamous cell carcinoma lesions. Kakefuda et al. (1967) were able to demonstrate virus-like bodies in the mitochondria of Shope papillomas.

b) Classification and Histogenesis of Tumors: The histogenesis of chemically induced malignancies in mouse skin commenced with a progressive localized alteration of the epithelium, initially by hyperplasia. This passed slowly into the formation of a benign tumor, and the latter gradually into a carcinoma (Deelman, 1923). Not only were more recent authors in agreement with this view on the formation of malignancies (Glucksmann, 1945; Wolbach, 1951; Dammert, 1961 and Siegler and Duran-Reynals, 1962), but Rous (1960) asserted in addition that the histogenesis was identical, irrespective of whether the carcinogen was a physical, chemical or biological agent. Early tumors probably developed from a central point (Madsen, 1965) or from the fusion of closely set multiple foci (Zackheim, 1963). Each tumor grew by multiplication of its own abnormal cells rather than by the continuing conversion of normal cells (Bauer, 1963). Hyperplasia of an area of skin was associated also with significant changes in the subepithelial elastic tissue. According to one theory, invasion by the neoplastic cancerous epithelium apparently began at points of greatest damage to the dermal elastic tissue (Willis, 1944). There was a simultaneous infiltration by leucocytes (Willis, 1945).

Hair follicles were recently implicated in playing a major role in epidermal carcinogenesis (Wolbach, 1951; Whiteley and Ghadially, 1952 and Giovanella and Heidelberger, 1967).

Chemical carcinogenesis induced benign epithelial tumors of the

papillomatous type more frequently than outright carcinomas, that is, the malignant form. Within the lifetime of an animal, papillomas developed, matured, regressed and disappeared, leaving little or no scarring. Benign tumors tended to grow more slowly and erratically than malignant tumors. Malignant lesions invaded neighbouring tissues whereas benign lesions did not. Benign tumors never metastesized; in contrast, malignant tumors were capable of spreading systemically. Haemorrhages and areas of necrosis were a common feature of the histological structure of malignant lesions. Benign tumors exhibited little tendency to haemorrhage or to produce areas of necrotic tissue (Walter and Israel, 1965).

Only three cell states can be distinguished with certainty; the normal, the benign and the malignant phases. Further classification not only becomes less precise, but also less meaningful. A disadvantage of introducing a further classification into this system is that tumor growth, like cell renewal systems, is continuous, and an attempt to define any further arbitrary stage, or a series of stages, tends to destroy the concept of continuity. Moreover, classification systems are artificial, and contain sources of error. Also, the probability of discovering two identical tumors is very low.

Owing to these difficulties, one must keep in mind when discussing such systems, the factors that such a classification inherently is disposed to conceal; growth is continuous; the system is artificial; no two tumors are alike, and there are errors in the system.

Induced benign papillomas of the mouse epidermis were classified into four categories (Shubik et al., 1953); (1) sessile papillomas, which were small and poorly keratinized; (2) pedunculated papillomas, large hyperkeratotic tumors connected to the skin by a thin stalk; (3) conical tumors, keratinous horns growing from a solid conical base,

and (4) carcinomata, with an indurated and elevated edge, and growing rapidly. This tumor was the only one that infiltrated the underlying tissue. This system is based on the morphological appearance of the mature tumor.

Another method of arriving at a classification was to categorize stages in the tumors development, as Ghadially's (1961) system, comprising five tumor types: the Bud-shaped Keratoacanthoma (type 1) (KA_1); the Dome-shaped KA2; the Berry-shaped KA3; the Sebaceous Gland Adenoma type 4; and the true benign papilloma (type 5).

In the following section, the developmental stages of these tumors are described in relation to the simplified, symbolic diagrams presented in Figure 4.

Figure 4 represents a portion of epidermis with a few active hair follicles (Anagen VI). The light stipling indicates the keratin, the dark stipling the remaining of the epidermis.

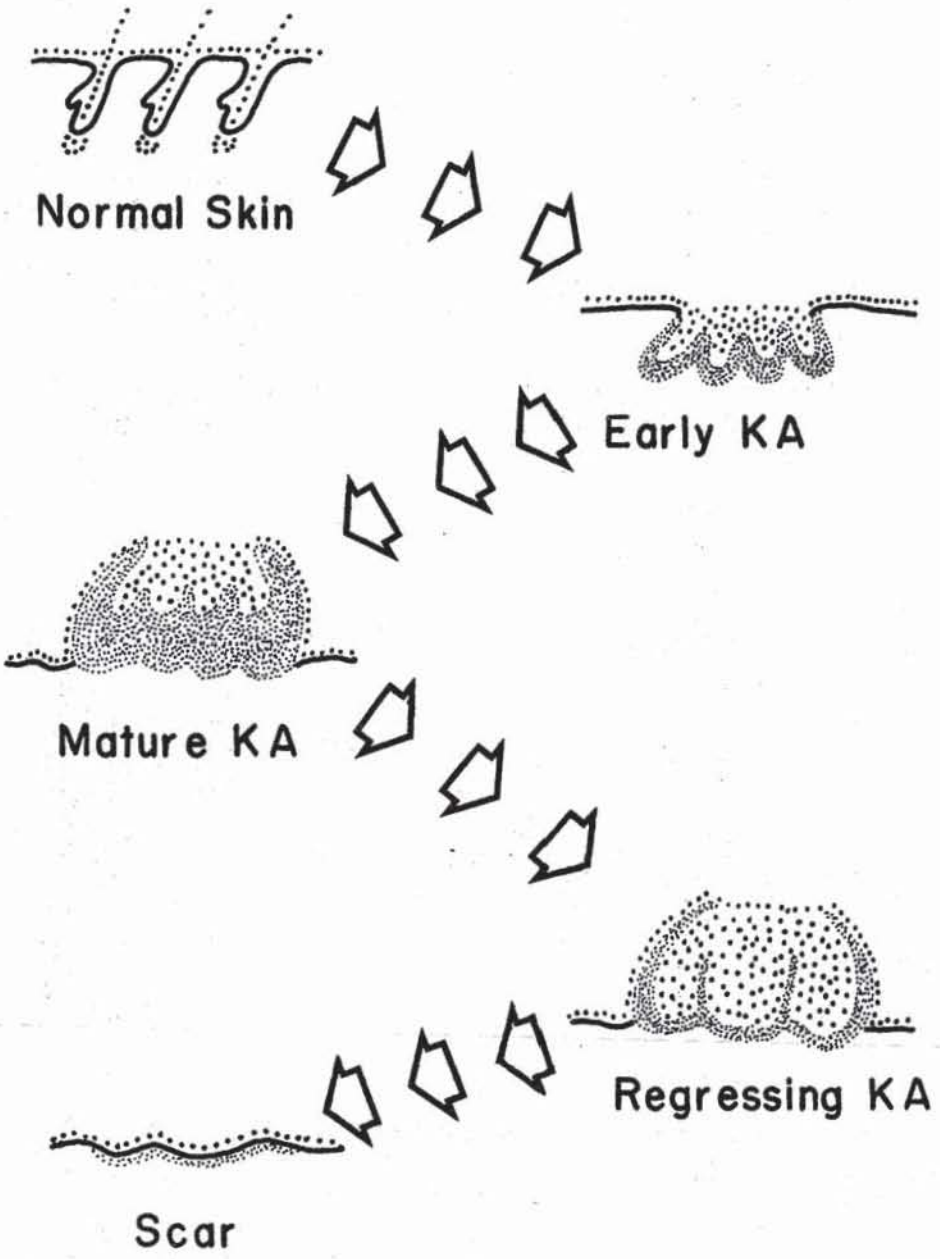
The bud shaped keratoacanthoma type I developed from the walls of the adjacent hair follicles. This soon formed a small tumor (early KA) which was characterized by a thickened and elongated follicular epithelium. The sebaceous glands and deeper portions of the follicle did not participate in the genesis of this lesion.

At a further stage, the mature form of the KA developed. Macroscopically, the lesion at this stage had a compact and well defined rounded appearance. Microscopically the tumor consisted of a cup of epithelium containing irregular papillary processes, the remnants of the follicular walls, and a central keratinous mass. The external walls were smooth and devoid of hair.

The turning point at which a mature KA became a regressing KA is difficult to decide. The regressing KA may be identified by an

Figure 4

Histogenesis of Keratoacanthoma



overabundance of keratin contained within the thin walled epithelial cup. Thus, the mature KA contains up to equal amounts of epithelium and keratin while the regressing KA is composed of more keratin than epithelium. The regressing KA has often been observed to degenerate into a small scar. The preceeding discussion was based on a classification by Ghadially (1961) and modified after the observations reported by Chivers (1967).

A variety of abnormal features have been observed in the ultrastructure of experimentally benign tumors. The arrangement and distribution of cytoplasmic organelles were disorganized. Focal areas of disintegration occurred as well as discontinuities in the basal membrane (Frei, 1962).

Melanoma is a malignant tumor derived from the melanocyte (Attie and Khaliff, 1964; Pinkus, 1966). The arising tumor may be melanotic or amelanotic (Robbins, 1967, Lever, 1967), containing more or less melanoma granulations, or none at all. Malignant melanomata were not observed in this study, and a specific search for amelanotic melanoma was not carried out.

An association between intra-epidermal carcinoma (Bowen's Disease) with cutaneous and systemic cancer has been demonstrated by Graham and Helwig (1965). Such lesions were characterized by hyperkeratosis, inflammatory infiltration in the upper corium, a striking loss of normal polarity, and/or progression of epidermal cell maturation. Atypical epithelial cell proliferation occurred, as well as vacuolated cells with abnormal mitotic figures at all levels of the epidermis. The lesions were delimited by a basement membrane at the dermo-epidermal junction. From the examination of serial sections of human Bowen's

lesions excised from hair-bearing areas, these authors demonstrated that all layers of the outer root sheath of the pilosebaceous follicle were involved. The atypical cellular proliferation occurred in the sebaceous glands and in part of the outer root sheath. However, the cells of the hair follicles below the level of the sebaceous glands remained unchanged.

An ultrastructural investigation of human Bowen's disease demonstrated a discontinuity of the basement membrane through which were observed cytoplasmic projections of epidermal cells. The intercellular spaces were widened and the number of intact desmosomes was decreased. The nucleolar size was enlarged; virus-like particles were also noted (Olson, 1968).

With epithelial tumors, the malignant state may eventually culminate in the destruction of the basement membrane, and in a transformation of the cell population into anaplastic cells which may invade the deeper connective tissue and muscle.

Anaplasia is characterized by loss of cell differentiation, organization and specific function (Dorland, 1965). In the anaplastic classification scheme, the degree of differentiation is thus the key characteristic. Epithelial pearls occurred in the well differentiated squamous cell carcinomata. Such epithelial pearls were composed of groups of cells with a central whorl of keratin, surrounded by one or several layers of prickle cells, and sometimes by a poorly formed basal layer. Cells of a less differentiated tumor contained keratin although groups of prickle cells may be still present. The highly undifferentiated tumor exhibited very poor organization; the cells varied greatly in size and shape and giant-sized mitotic figures were present. A scanty but vascular stroma supported the diffuse layer of neoplastic cells (Walter and Isreal, 1965; Anderson, 1966).

Although papillomas are derived from regenerating epithelium, squamous cell carcinomata arose in atrophic neighbouring epithelium, and not directly from papillomas (Siegler and Duran-Reynals, 1962). Zackheim (1964) graded the squamous cell carcinomas of rat epidermis according to depth of penetration and degree of anaplasticity. The grades according to depth were: A, restricted to the upper one-third of the cutis; B, involving the upper two-thirds of the cutis; C, the entire cutis was affected; and grade D, where the underlying muscle layer, the panniculus carnosus, became invaded by tumor tissue. The classification indicating the degree of anaplasticity was similar. This author also demonstrated that after one application of 0.18 ml. of 0.5% DMBA in acetone, a mild inflamatory reaction began on the third day. Later, most of the sebaceous glands disappeared and the follicular walls had undergone varying degrees of degeneration. Two weeks after the initiation of weekly applications of the carcinogen to the unshaved midback region, alopecia was observed (alopecia: the absence of hair from skin areas where it is normally found [Dorland, 1965]). During the third and fourth months after the beginning of the carcinogen application, the treated epidermis was hyperplastic. Progressively more cells became malignant, and eventually extensive portions of the epidermis were replaced by malignant cells. Treatment beyond six months did not augment the tumor yield. The authors assumed these rat squamous cell carcinoma to have arisen from the epidermis proper.

Frei (1962) noted that the basement membranes became discontinuous when a malignant tumor was present. Cells projected between the gaps in several places. Bundles of collagen fibres were observed among stratum granulosum cells.

The basal cell carcinoma or rodent ulcer is a tumor which rarely metastesizes (Lakshmipathi and Hunt, 1967), although it slowly invades surrounding tissues (Walter and Israel, 1965). Histologically, such tumors were composed of nests of closely packed, oval shaped cells of uniform size. The dark nuclei were separated by scanty amounts of spineless cytoplasm. The nests were frequently rimmed by a single layer of similar cells arranged in radial pattern (Anderson, 1966).

The histogenesis of the basal cell carcinoma has been subject to considerable discussion. Teloh and Wheelock (1949) postulated that the basal cell carcinoma arose from the basal layer of the epidermis, the sheath of the hair shaft, the pilar papilla, or occasionally from the sebaceous glands. In a study of the human basal cell carcinoma, Smith and Swerdlow (1956) proposed that the tumor developed from the hair primordium or "Anlage". Swerdlow (1958) suggested that these tumors were an aberrant exaggeration of normal hair development, cyclic regrowth and regeneration. He indicated further that these carcinomas tended to pinch off stroma much in the manner as does the papilla during the early Anagen stages.

A common identification error was to separate the baso-squamous carcinoma from the basal cell carcinomata. The baso-squamous carcinoma was, in fact, a basal cell carcinoma containing foci of squamous cells or epithelial pearls. Occasionally the epithelial pearls became calcified in the center of the nests of basal cells (Anderson, 1966).

After painting the backs of rats with anthracene or MCA in acetone three times weekly for six months, Zackheim (1962) demonstrated that the first histological sign of a basal cell carcinoma was a local proliferation of cells situated in the posterior surface of the follicular ostia, that is, at the obtuse angle with the epidermis. This cell group

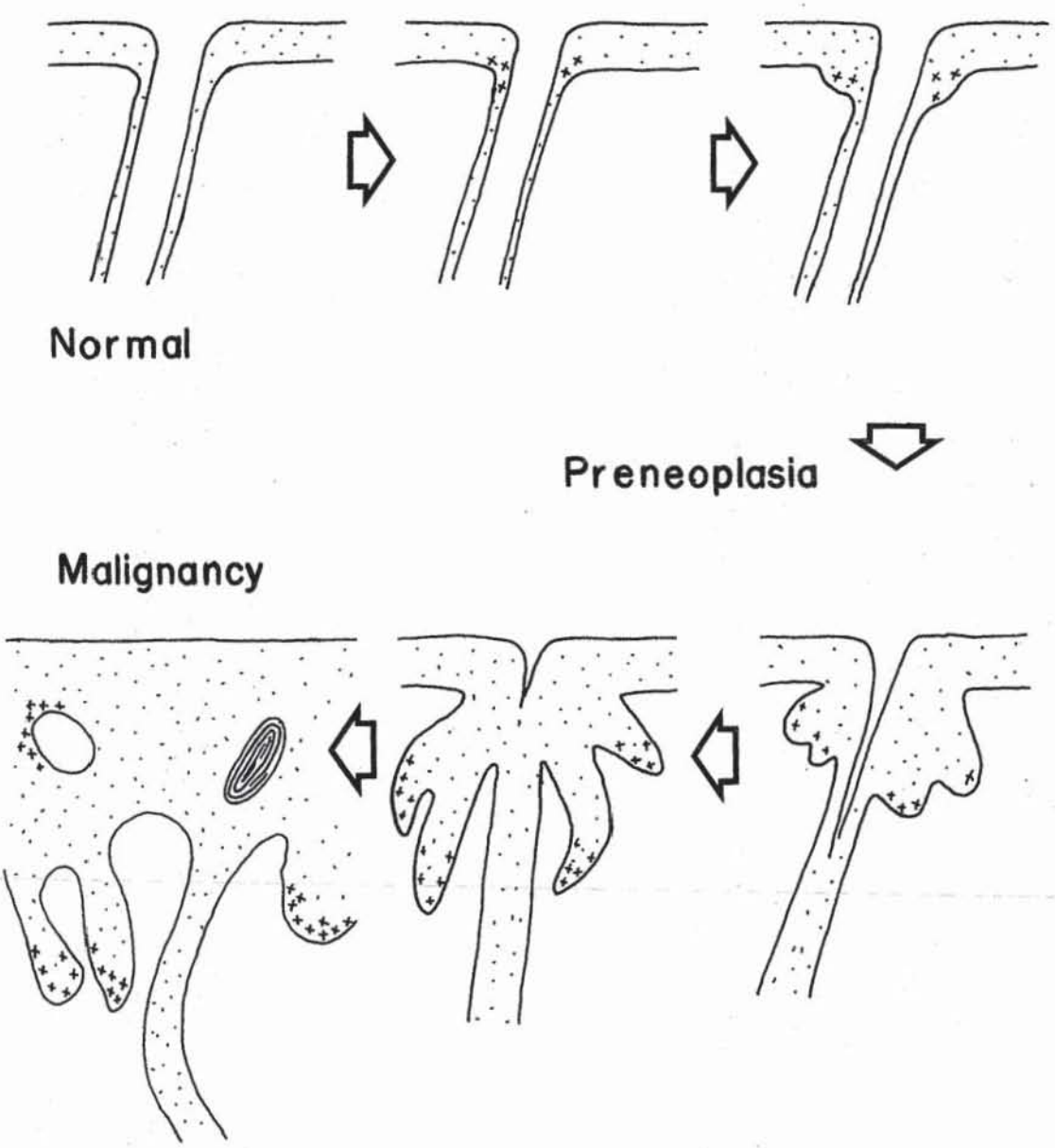
gradually progressed to enclose the entire ostia and to culminate into a funnel-shaped tumor.

As early as seven days after a single application of MCA in benzene to the skin of female SWR mice, Liang (1948) noted foci of cells at the junction between the basal epidermis with the hair follicle. These cells multiplied extensively and were composed of groups of cells that were either closely packed or loosely dispersed. If painted with benzene there was no basic change.

The cells near the follicular ostium have also been implicated in the histogenesis of benzopyrene induced squamous cell carcinomas in albino mice (Chivers 1967, 1969). Such lesions were initiated by a localized proliferation of cells in the follicular ostia (Fig. 5). This was followed by a down growth of a cord of epidermal cells. At about this time, the original follicle walls became indistinguishable from the surrounding epidermal tissue. Finally fusion of cords from neighbouring follicles occurred, and the typical form of the squamous cell carcinoma became apparent. Niskanen (1962) ascertained that a single intragastric instillation of either urethan, MCA or DMBA without further treatment, resulted in an irregular epidermal hyperplasia, which was particularly noticeable in the perifollicular regions. In this area, the cells were elongated and arranged in several compact layers. Disturbances of keratinization, destruction of hair follicles, atrophy of the sebaceous glands, and a slightly enhanced mitotic activity were also noted. Local exposure of the skin to a dipole type promoting agent caused a regularly arranged hyperplasia of the IFE while the pilosebaceous apparatus remained preserved. When both initiator and promoter were applied to the skin, areas of irregular hyperplasia and follicle damage alternated with areas containing a more regular

Figure 5

Histogenesis of Benzopyrene Induced Mouse Squamous Cell Carcinoma



xxx indicate areas of enhanced mitotic activity.

hyperplasia of the IFE and preservation of the pilosebaceous units.

Albert et al. (1967a) determined that radiation-induced follicular damage was manifested by follicular atrophy and a loss of follicles. The chronic follicular damage was an important factor in the initiation of epithelial skin tumors in the rat.

Albert et al. (1969) demonstrated that tumors formed periodically after initiation by irradiation. They also stated that the tumors arose from atrophic follicles.

Albert et al. (1967b) ascertained further that tumor initiation was dependent on the penetration of electrons into rat skin during Telogen. Thus, tumors were initiated from cells at some levels higher than the bottom of a Telogen hair follicle. These same authors (1967c) further postulated that skin tumor induction depended upon the size of the area irradiated.

c) Cytokinetics: The epidermis is a dynamic cell population where a constant flow of cells passed from the proliferating cells in the basal layers to the surface of the epidermis. Lajtha (1960) stressed that during cytokinetic studies, it must be ascertained whether there are: 1. population changes, 2. population shifts, 3. cell cycle changes caused mitotic delay, 4. changes observed within one interphase, and 5. pool changes.

Early authors recognized that there occurred considerable cell damage to this dynamic tissue after carcinogen application (Page, 1938, Hamperl et al., 1943, and Glucksman, 1945). Indeed, Cramer and Stowell (1942a, 1942b) suggested that this was an immediate effect of the carcinogen on the skin epithelium.

The normal rate of cell death was 0.6% of the total cell mass in

the epidermis per hour (Iversen, 1964). After a single application of MCA in benzene there occurred an increased rate of cellular death for a few days.

After a single application of various concentrations of DMBA (range 1 μg to 10 μg) to the back IFE of female STS mice, aged 6 - 8 weeks, during Telogen, Hennings and Boutwell (1969) demonstrated that there occurred a period of inhibition of DNA synthesis. It lasted at least 30 minutes. They did not observe any relationship between the degree of inhibition and the number of tumors which were thus induced. Hennings and Boutwell (1968) also ascertained that croton oil reduced the amount of DNA synthesis by 60%, 9 hours after its application, and that there was a rebound reaction to about three fold over normal by 18 hours. On the other hand, RNA activity was two fold normal by 6 hours after croton oil application and 2 1/2 fold at 12 hours. Twenty-four and 48 hours later there still remained increased levels of DNA and RNA synthesis.

Shimkin et al. (1969) suggested that inhibition of DNA synthesis, as estimated by the labelling index, may represent a toxic side effect rather than being a key event in carcinogenesis. Raick (1969, personal communication) reported that the inhibition of DNA synthesis, estimated by the specific activity of DNA, was longest when initiated by higher concentrations of the A_1 fraction of croton oil. Phenomena which could be interpreted as rebound synthesis and partial cyclic activity were also observed.

The generation time (tC) was ascertained to be similar whether measured of fibrosarcoma of C_3H mice in vivo or in vitro , that is, approximately 17 hours. The growth rate was depressed in vitro because of increased cell death. About 24% of cells were synthesizing DNA at

any one time (Frindel et al., 1967). In a variety of tumors measured in vitro, Frindel et al. (1968) determined the duration of S phase to range from 7 to 19 hours and generation times of 1 to 4 days. Human squamous cell carcinoma cytokinetics were estimated after administration of vinblastine sulfate (VLB); tC was 6 to 9 days, tM was 17 to 92 minutes and tS was 4.6 and 39 hours (Meyer, 1969).

Malignant tumors arising from the glandular cells of the human breast exhibited a tC = 33 hours. Two estimates of tC of human basal cell carcinoma were 67 and 97 hours, while a tC of 32 hours was noted for epidermoid carcinoma in man (Bresciani, 1968). The generation time of five human solid tumors were ascertained after the biopsies were maintained in tissue culture. Tumors described as basal cell carcinoma, epidermoid carcinoma and spindle cell carcinoma in vitro exhibited a tC between one and four days (Frindel et al.,1968). Cytokinetic studies by Bennington (1969) on invasive squamous carcinoma of human cervix revealed that the parameters of growth of this type of squamous cell carcinoma in vivo after a pulse label of H^3-T were tC = 14.3 to 15.5 hours, tG_2 = 1.1 to 1.5 hours. The generation time of epidermal carcinoma induced by MCA in the mouse was tC = 32 hours (Dormer et al., 1964).

Mendelsohn (1962) ascertained that in spontaneous breast cancer 40% of all cells in the C_3H mouse were in the mitotic cycle at any one time (i.e. N = 40%).

The same tumor under similar conditions exhibited a tC = 1 to 3 1/2 days; tS = 9 to 13 hours; and tG_1 = 1 to 4 hours (Mendelsohn et al., 1960).

In the variety of tumors studied by Frindel et al. (1968), the tS ranged from 7 to 19 hours. Reiskin and Mendelsohn (1962) determined

that tS of hamster cheek pouch epithelial cells varied less from tumor to tumor than it did from the normal hamster cheek pouch to cheek pouch. In a study of carcinogen induced heptoma cells Post et al. (1964) concluded that both the replication time (tC) and DNA synthesis times (tS) were longer than that of the non-malignant cells.

5. Growth Controlling Mechanisms

"One cannot expect the growth of such a heterogenous composite as an eye or a limb, let alone a whole animal, to conform to any single monotonic mathematical function. An emperical growth curve is but an enveloping blanket, covering underlying changes as diverse as are the parts under consideration, some of which increase, while others remain stationary, and still others regress, each according to its own peculiar time schedule and rate" (Weiss and Kavanau, 1957). Accordingly, if one can derive observations on growth from an inductive growth curve, such observations become valid only if they are in fact verified. A number of models can stimulate the general sigmoid shape of growth curves, e.g. auto-catalytic monomolecular reactions (Robertson, 1923), masses subject simultaneously to surface gains in proportion and proportional volume losses (Rubner, 1902; L. Bertalanffy, 1942), or else negative feed back mechanisms (Weiss, 1957).

a) Factors affecting growth: A functional interplay involving a tissue specific inhibitor of mitosis (chalone) was postulated to occur between basal cells and differentiating cells in the mouse epidermis (Bullough and Laurence, 1960). Iversen and Bjerknes (1963) proposed a cybenetic theory involving that interaction. More recently, work was concluded attempting to coordinate both views (Bullough, Laurence, Iversen and Elgjo, 1967).

Other factors are likewise important in the control of growth mechanisms. Among these are an adequate supply of metabolites from which the energy for mitosis was derived (Loeb et al., 1930). The mitotic index of epidermis was demonstrated to bear an inverse relationship to the level of blood sugar available (Bullough, 1949a). Poisons of glucose metabolism, such as phloredzen, depressed the mitotic index. Subcutaneous injections of starch increased the index while injections of insulin (thus increasing glucose utilization) depressed the mitotic index (Bullough, 1949; 1950a; 1950b). These apparent contradictions remain yet to be explained.

Starvation, or a mere reduction of diet, yielded a lowering of the blood glucose level as well as a decreased mitotic index. However, the cell population of the epidermis and sebaceous glands remained unaltered under these conditions (Bullough, 1949; Bullough and Eisa, 1950; Bullough and Ebling, 1952).

Adrenalectomy did not affect the level of blood sugar but it did alter mitotic activity (Green and Ghadially, 1951).

Moreover, direct relationship was ascertained between mitosis and oxygen tension (Medawar, 1947; Bullough and Johnson, 1951).

The principal pathway for glucose utilization by epidermal cells was the Krebs cycle. Most of the respiratory substrate of the skin consisted of lipids (Gilbert, 1962). The energy production of cells in vitro was determined to occur via the carbohydrate metabolism, of cells in vivo, about 70% was by means of fatty acid metabolism (Cruickshank, 1965).

Basic requirements for mitosis have been demonstrated to be adequate carbohydrate and oxygen supplies in particular. Although much

in vivo energy supply in the epidermis is through lipid metabolism, it is unknown whether this supply is utilized also in the mitotic process.

Loeb and Haven (1929) demonstrated in guinea pigs that a correlation existed between epidermal mitotic activity and the estrous cycle. Once the animals attained maturity, the mitotic activity of females was generally lower than that of males. Bullough (1946) ascertained that the mitotic rhythm associated with the estrous cycle was caused by the particular estrogen concentration. The mitotic rate was enhanced while the duration of mitosis was decreased after estrogen administration.

Blumenfeld (1939) observed that ovariectomy resulted in a diminuation of the thickness of the adrenal cortex. Fisher (1967) interpreted this to imply that the ovarian hormones in the intact animal might have excerted a stimulatory effect on the adrenal cortex, and caused the secretion of a higher level of adrenal-cortical hormones; the latter principles have since been shown to be involved in mitosis inhibition.

Estrogens have been confirmed to be general mitotic stimulators (Epifanova, 1958; 1961; 1962). Yet, apparently only the interphase cell was effected by estrogens. In specific and non-specific tissues, the effect was different. Epifanova (1964, 1966) suggested that the number of cells capable of division was increased in all tissues but to a greater extent in those tissues where estrogens normally excerted their specific effects.

Injection of testosterone propionate stimulated mitosis in the testis of minnows (Bullough, 1942b). Administration daily of 1.0 mg. testosterone propionate resulted in an increase in size and activity of sebaceous glands (Ebling, 1948). Castration of normal male mice depressed the epidermal mitotic rate and shortened the mitotic duration.

Subcutaneous injection of testosterone propionate succeeded in rectifying these parameters to normal levels (Bullough and van Oerdt, 1950). Moreover, Ebling (1957) ascertained that testosterone enhanced the rate of cell loss from the epidermis.

After application of an aqueous adrenal extract, Baker and Whitaker (1948) noted a thinning of the epidermis. Bullough (1952a) determined that cortisone administration delayed the vascularization of wounds, weakened the primary healing response, depressed cellular proliferation, and retarded tissue reorganization. Administration of gluco-corticoids to rats over an extended period caused a thinning of the IFE, arrest of hair growth, and further, it reduced the size of the sebaceous glands (Castor and Baker, 1950). Noting that 24 hours after an injection of cortisol hemisuccinate there was a decline of the mitotic activity of IFE. Davis (1964) concluded that cortisol did not affect mitosis directly but rather the entry of cells into mitosis. Chaudhry et al. (1956c) ascertained that corticosteroids blocked mitosis by acting on interphase. Cortisone also inhibited mitosis in regenerating liver (Roberts et al., 1952; Bullough, 1965). These authors suggested that part of this occurrence was brought about by a partial blockage of DNA synthesis.

A circadian rhythm of mitotic activity was demonsrated in the epidermis of mice (Halberg et al., 1959). Thus, the anti-mitotic action of adrenaline may be partly ascribable to the release of cortisone via the pituitary pathway (Bullough, 1952a).

Other biological substances were demonsrated to exert likewise an effect on cell division. Injection of kidney extract from a young embryo into an older embryo resulted in an increase of the renal mitotic

rate. However, differentiation was not affected appreciably when an extract of the homologous organ from similar aged animal was employed (Weiss, 1952). This suggested that each tissue contained a specific substance or substances by which that tissue's growth was governed. Saetren (1956) ascertained a similar tissue specificity of liver and kidney extracts. Teir (1952b) claimed that this control became lost when a tissue turned malignant. Bullough (1965) suggested that the stimulatory effect of homogenates could be ascribed to a slight damage of the cells resulting in loss of chalone (the specific inhibitory substance). Katayama (1957; 1958) and Epifanova (1961) demonstrated that ATP alone did not affect mitotic activity.

X-irradiation depressed mitotic activity (Smith, 1951). The extent of radiation seemed not to affect the rate of depression, but high doses decelerated recovery (Knowlton et al., 1948).

Bullough (1962) reported that acetylcholine did not influence the mitotic activity. Others (Kulagin 1959a, 1959b, 1960; Overton, 1950) have suggested that a mitosis stimulating substance was produced by the nervous tissue. Thus, sympathectomy was followed by a decreased mitotic activity in epidermis (Friedwald and Busche, 1944).

Cycles of daily mitotic activity were demonstrated by Ortiz Pecon (1933) to occur in mouse epidermis. Diurnal variation in the mitotic activity were reported likewise in the ear epidermis of mice (Cooper and Franklin, 1940). This rhythm was just the reverse of that in the human infant prepuce (Broders and Dublin, 1939). Scheving (1959) demonstrated a complete circadian rhythm of mitotic activity in human epidermis although he included in the mitotic counts several types of apparently non-dividing cells such as melanocytes, lightly pigmented

cells and cells with heavily granulated nuclei.

The mitotic diurnal cycle of mouse ear and cornea was inversely related to the rhythm of the animal's activity (Bullough, 1948a; Basama and Vasama, 1958). Similarly, the rhythm was inversely related to the blood sugar level (Bullough, 1949c).

By reversing the lighting schedule to which mice were subjected, Halbert et al. (1957) was able to achieve a slow eventual reversal of the cycle of mitotic activity. These authors concluded from that observation that the prime cause of the diurnal cycle of mitosis was light. The mitotic diurnal cycle may be brought about by a variation in the rate of mitosis as well as in the duration of mitosis (Bullough and Laurence, 1966b).

Bertalanffy et al. (1965) determined that the mitotic rate of rat IFE increased with age. Similar observations were reported by Thuringer and Cooper (1950), Meyer et al. (1956) and Thuringer and Katzberg (1959). Bullough (1949c) reported that the mitotic index of the epidermis of male mice exhibited peaks in immaturity as in old age, and demonstrated low mitotic activity both during maturity and old age.

The dermal connective tissue was involved in the epithelial control of growth to a greater extent than merely by supplying nutrients. Clinical injection needles frequently deposited in man epithelial fragments subcutaneously, intramuscularly or intravenously; however, these fragments did not survive in the displaced position (Little, 1955; Gibson and Norris, 1958). Human epidermal epithelium implanted subcutaneously likewise did not remain viable (van Scott and Reinerton, 1961).

b) Cybernetic Control

A cybernetic system is a dynamic mechanism maintaining an equilibrium by means of a negative feed back (Iversen and Bjerknes, 1963). L. Bertalanffy (1960) suggested a model of growth by assuming that a cell cannot exist beyond a certain critical size. In this model, when a cell contains a critical size it must either divide or stop growing. The feedback mechanism in this model is not an inhibitory one but rather one of principle. Although the model is useful it cannot account for phenomena such as regeneration.

Weiss and Kananau (1957) proposed another model based on cybernetic principles. They suggested that not only did each cell type produce compounds characteristic of that cell to act as catalysts, but also that each cell produced antagonists to these catalysts, and is thus capable to inhibit the production of catalysts. Thus, differentiated cells would produce an inhibitor that diffused into the surrounding medium and regulated the activity of cells in proliferative and differentiating compartments. In this model hyperplasia is explained by a loss of inhibitor followed by subsequent rapid regenerative response.

Szent-Gyorgyi (1965) extracted a substance he called retine, which restricted mitotic activity but was neither tissue nor species-specific. This substance was a methylglyoxal derivative of small molecular weight. Bullough and Laurence (1966a) postulated that "this is particularly interesting since it has been known for a long time that cells contain a powerful enzyme system for transforming methylglyoxal into lactic acid, but this enzyme system has never previously been fitted into the metabolic framework". As retine is "bound in vivo to a hydrophilic colloid", (Szent-Gyorgyi, 1965) it may not exist free within the cell, and when released may become transformed to lactic acid before it can perform

any extracellular action.

In addition Szent-Gyorgyi (1965) isolated promine, a growth-stimulating factor. Although little is known of that substance, it might fit into the machinery of homeostasis, and may in fact be required to explain the stimulatory action of tissue extracts, as they were reported by Needham (1960), for instance.

As it is evident, growth is not a simple phenomenon and probably has many modes of control. The following section reviews one of the likely control mechanisms which is showing particular promise.

Lysosomes are widespread in mammalian epidermal cells (Diengdoh, 1964). "Among the many homeostatic mechanisms operating on a subcellular level there may be those that control accessibility of cell constituents and the acid hydrolases segregated in lysosomes" (Novikoff, 1961). It seems also conceivable that the chalone mechanism acted to stabilize the lysosomes membrane, thereby delaying mitosis and prolonging cell life (Bullough and Laurence, 1966). The cell membrane is another factor which must be involved in homeostasis. For if it were not, phenomena as the synchronous division of synctial cells, or of multinucleated giant tumor cells, would in all probability not occur.

After wounding, the disrupted IFE was formed by migration of cells from the wound edge, and subsequently thickening by mitosis (Gillman and Penn, 1956; Johnson and McMann, 1960). The mitotic activity of the epidermis became greatly enhanced after wounding (Blumenfeld, 1943). Bullough and Laurence (1960a, 1960b) determined on mouse ear epidermis that a peak of mitotic activity developed within one millimeter of the edge of the wound. Some authors (Davidson, 1943) suggested that this was brought about by the production of a "wound hormone". But the

existance of a "wound hormone" was disputed when Bullough and Laurence (1960a) demonstrated that a wound which was inflicted from the inside outwards, and thus did not injure at all the epidermis, failed to effect the mitotic activity of the IFE; and this despite the circumstance that there occurred a burst of mitosis in the injured areas, that is, of the cells of hypodermis, hair follicles, and the sebaceous glands. Thus, if a wound hormone existed it must of necessity have been tissue specific. Further, Bullough and Laurence placed an incision into mouse ears in such a manner that the wound did not penetrate the central plate of cartilage. This resulted in a one mm. wide zone of increased mitotic activity around the edge of the wound. But in the intact epidermis on the opposite side of the ear there also occurred a similar zone of higher mitotic activity. All augmented mitotic activity was within a radius of one millimeter from the center of the wound. These observations could be interpreted either that a stimulating "wound hormone" existed with a radius of action equalling one millimeter, or else that a tissue specific mitotic inhibitor normally present in the IFE diffused from the wound for that distance in all directions. To test which hypothesis was true, Bullough and Laurence (1960a) removed a piece of IFE, 3 mm. square from one side of a mouse ear. If the increase of mitosis after wounding was brought about by "wound hormone" then there should have occurred on the opposite side of the IFE an increased mitotic activity opposite the edges of the wound and a center of low or unchanged mitotic activity opposite the center of the wound, as the action of the "wound hormone" had a radius of one millimeter. But if the increase in mitosis after wounding was caused by a lowered concentration of mitotic inhibitor, the maximum of mitotic

activity should occur opposite the center of the wound. The observations confirmed the latter possibility and provided evidence for the presence of an epidermal mitotic inhibitor substance.

This inhibitor, designated the "chalone" by Bullough, is tissue specific, and its concentration depends upon the state of the tissue (Bullough and Laurence, 1960b). A crude extract apparently of protein nature has been isolated (Bullough, Hewitt and Laurence, 1964). Other authors characterized it as being possibly a basic glycoprotein with a molecular weight of approximately 25,000 (Homan and Boldingh cited by Bullough, Laurence, Iversen and Elgjo, 1967). The granulocytic chalone was reported to be a biologically highly active organic compound with a weight of about 4000 (Rytomaa, 1969). That author suggested that the chalone of granulocytes inhibited both DNA and RNA synthesis. Chalone was found to be effective only when it was complexed with adrenaline (Bullough and Laurence, 1964a). The action of the adrenaline-chalone complex apparently acted by inhibiting cells just prior to division (Bullough and Laurence, 1964b). Recently it was determined that low concentrations of hydrocortisone alone did not excert any anti-mitotic effect, but when present with adrenaline and chalone prolonged their action, although it did not enhance the inhibitory effect of these compounds (Bullough and Laurence, 1968c).

Rytomaa and Kiviniemi (1969) proposed that the primary action of hydrocortisone might be on the cell membrane. Hydrocortisone possibly altered the permeability of the cell membrane to chalone, thereby reducing the rate of loss of chalone from the cells.

Although there is a loosening of mitotic control in the chalone theory, other factors were likewise implicated. For example, denerva-

tion reduced the incidence of mitoses in wounded and unwounded tissue (Kulagin, 1958b, 1960). Numerous authors, including Bertalanffy et al. (1965) demonstrated the occurrence of not only a continued diurnal rhythm after epidermal wounding but also that this rhythm was more pronounced than in the intact cell population.

By stripping the stratum corneum from human forearm Pinkus (1952) ascertained that a rise in the number of mitoses began 24 hours later and reached a mitotic peak by 72 hours. Other authors (Lobitz and Holyoke, 1954), employing a similar technique, determined that glycogen accumulated in the epidermal basal layer after injury, and the glycogen disappeared as the peak of mitotic activity was attained.

Fisher (1967) concluded from available evidence that the reaction of the epidermis to wound infliction proceeded in three steps: i) loss of chalone resulting in a cessation of mitotic inhibition; ii) glycogen became accumulated in the basal cells, and iii) cell division occurred utilizing the energy provided by the accumulation of glycogen.

Chalone was shown not to inhibit the synthesis of DNA; it was concluded from that observation that it probably acted at a stage later than S phase (Baden and Sviokla, 1968). If chalone in fact accumulated cells between the S phase and mitosis, then these cells are likely held in a G_o phase derived from G_2; perhaps the action of the chalone system is to hold cells in the former phase temporarily. The circumstance that some cells were in fact in a G_o phase derived from G_2 was recently demonstrated by Frankfort (1967).

c) Growth Control in Tumors: In malignant tissue with high mitotic activity, a diurnal rhythm of mitotic activity was absent (Dublin et al., 1940; Golobiva, 1958; Chivers, 1967).

It is feasible to define tumor growth by estimates of augmentated cell number; yet, such estimates are applicable to very few tumors. Other parameters that were employed were: caliper measurements of diameters of superficial tumors (Blum, 1943); radiographic measurements on X-ray films when lesions displaced bone (Finkel et al., 1961), lung (Schwartz, 1961) or soft tissues (Ingleby et al., 1958). Such types of estimates cannot exclude several extraeous factors, such as hemmorrhage or stroma, which may become changed with the progression of growth of the tumors (Mendelsohn, 1963).

Conducting a comparison between the cytokinetics of normal basal epithelium and induced epidermal carcinomata in the hamster cheek pouch, Reiskin and Mendelsohn (1962) determined that tS varied more widely between normal epidermis than between the tumors derived from that tissue. Mendelsohn (1963) suggested that the tumor cell population displayed less variability conceivably because it was not subject to the many (and often poorly understood) control mechanisms affecting the normal steady-state tissues.

In instances, tumor cells proliferated faster than the cell population of origin (Bertalanffy, 1967). However, the concept that "tumor cells do not necessarily proliferate faster than normal cells..... is gaining acceptance" (Baserga, 1965; Bertalanffy, 1967). Thus tumor growth likewise involves such factors as the size of the fraction of cells in the proliferative pool, and cell loss by migration of cell death.

The control of cell division appears to be to a large extent at least rather a problem of control of DNA synthesis, as a cell that has completed the S phase will eventually proceed to division (Baserga, 1965).

While the concentration of enzymes and precursors required for DNA synthesis are essential for the continuation of the S phase (Stone and Prescott, 1964), the role of these factors in the actual initiation of DNA synthesis still remains uncertain (Powell, 1962a; 1962b).

It is conceivable that one of the side effects of carcinogenesis is an inhibition of RNA synthesis which in turn may affect or inhibit DNA synthesis. Baserga (1965) suggested that the events in initiation of DNA synthesis were similar to bacterial enzyme induction, or in higher animals, to the action of estrogens (Hamilton, 1964). Baserga (1965) summarized the hypothetical sequence of events thusly: "an environmental stimulus, perhaps a critical concentration of deoxynucleotides or a slight modification in the physical state of the DNA molecule, may inactivate the repressor of a genetically inactive portion of DNA. The inactivation of the repressor would cause the synthesis of a template RNA which, in turn, would code the enzymes involved in the separation of the DNA strands and the polymerization of the component deoxynucleotides."

Some inroads to the problem of the control of cell division, as indirectly also to the control of the initiation of the S phase in tumors, have been made by applying the chalone concept. Using rabbits bearing the Vx2 epidermal tumor, Bullough and Laurence (1968a) ascertained that the intracellular concentration of chalone was reduced in the tumor cells. They concluded that the high rate of loss of chalone from the tumor lead to the inhibition of mitosis of the surface IFE. The tumor remained insensitive to administration of increasing concentrations of chalone. Yet, when pig epidermal chalone with adrenalin and hydrocortison was administered, the mitotic rate of the tumor became

depressed by 53%. Thus, not only was the chalone concentration reduced in the cells of Vx2 tumor, but its cells were less sensitive to it alone.

The Harding-Passey melanoma of mice and an amelanotic melanoma of hamsters were both shown to contain a substance with the characteristics of a melanocyte chalone (Bullough and Laurence, 1968b). Melanocyte chalone prevented mitosis in vivo as in vitro, but only of melanotic cells.

Granulocytic precursor cells were likewise demonstrated to be inhibited by a tissue specific chalone. The same chalone affected also chloroleukemic cells. But concentration of the chalone in chloroleukemic cells was much lower than in normal mature granulocytes, while concentration of the chalone in leukemic serum was greater than in similar non-leukemic serum. This signified that the chalone in that tumor was lost by the malignant cells to the surrounding medium (Rytomaa and Kivinemi, 1968).

RNA isolated from homolgous tissues inhibited tumor growth, while that extracted from other tissues did not (Aksenova et al., 1965). This supported the assumption (Frenster, 1965b) that the activating substance was a specific derepressor RNA which could become a hybrid with one strand of DNA allowing the complementary DNA strand to synthesize messenger RNA. A study of lymphocytes from calf thymus supported that possibility, (Frenster, 1965a), as well as the circumstance that RNA isolated from homologous tissue could in some cases inhibit tumor growth, while RNA separated from other sources was unable to do so (Aksenova et al., 1965).

6. Carcinogenesis

In a review of the early effects of hydrocarbons, that is, of chemical carcinogens, on mouse skin, Bock (1964) stated that "penetration of the skin by hydrocarbons is a passive process. It is controlled mainly by the solubility of the hydrocarbons in lipids and by the nature of the pathway by which they penetrate into the skin. The pathway in normal mice consists primarily of the pilosebaceous canal and associated sebaceous glands". He pointed out further that this pilosebaceous apparatus, as the pathway, were subject to considerable variations. In normal haired mice the penetration of epidermis by benzopyrene was not as rapid as in hairless mice. This was apparently because the follicles of the hairless mice exhibited much wider openings, and the sebaceous glands were larger. Pretreatment with various carcinogens caused atrophy of the sebaceous glands and hyperplasia of the IFE. After this pretreatment, BP penetrated the IFE even faster than before. Presumably this pretreatment altered the IFE in some manner to allow extensive and rapid penetration of BP through the entire area.

Sebaceous glands seem to be structures important to the initiation of tumors. In the skin of animals where sebaceous glands were absent, such as in newborn mice (Suntzeff et al., 1945) or epidermal burn scars (Lacassagne and Latarjet, 1946), tumors could be induced only with difficulty. Animals in which the sebaceous glands were destroyed, such as after painting with MCA in lanolin (Simpson et al., 1945) likewise did not develop tumors. It became evident, however, that destruction of the sebaceous glands was neither necessary nor sufficient for carcinogenic activity (Bock and Mund, 1958).

After painting the skin of mice with BP in acetone containing

1% mineral oil, Bock (1964) ascertained by a fluorescence technique that the maximal concentration of the carcinogen in the skin was attained within two hours. The last detectable free carcinogen was lost from the skin between 48 and 96 hours.

Carcinogen could accumulate in the sebaceous glands. When the glands broke down (within four days), a relatively heavy concentration of carcinogen became liberated to act on the cells of the follicular walls above the sebaceous gland level. More carcinogen would then be deposited in the area of the follicular ostium than at any other area of the epidermis which was not destroyed, as the carcinogen would accumulate in the small hills and valleys of the mouse's skin. Conceivably, this was a factor contributing to the circumstance that squamous cell carcinoma, following the administration of BP to the back skin of mice, developed from that specific locality more frequently than from the IFE proper (Chivers, 1967; 1969). Zackheim (1962) reported similar responses in the emergence of basal cell carcinoma after painting the back skin of rats with MCA in acetone.

The responses of the skin to local exposure of carcinogens, and to dipole tumor enhancers, are often quite different and sometimes even antaganistic. Such differences were summarized by Niskanen (1962) (Table 1).

The two main theories of carcinogenesis at present are: that cancer is brought about by a somatic gene mutation, and that neoplasia is a result of an extragenetic phenomenon. Neither possibility has been proven nor disproven, but the problem of cancer is so diverse that both theories may be involved during different situations (Bullough and Laurence, 1966b). Armitage and Doll (1957) suggested that the two stage

Table 1*

Differences between effect of initiators and of promotors in mouse skin

Criteria	Initiators	Promotors
1. Tumors	always develop and are independent of strain	development is strain dependent
2. Epidermal Hyperplasia	irregular and progressive	regular and lasts as long as the treatment
3. Pilosebaceous Apparatus	destroyed	intact
4. Cell Types	appearance of basal-type cells	increase of differentiating cells
5. Cell Morphology	increase in cellular and nuclear atypia and anaplasia	no atypia or anaplasia
6. Nucleo/ Cytoplasmic Ratio	increased	unchanged
7. Localization of mitosis	in all layers of nucleated cells	in basal layers only
8. Cell surface-to-cell surface contact	sticky connection	wide intercellular spaces
9. Keratinization (differentiation)	retarded or misguided	secondarily delayed

* After Niskannen, 1962.

theory of carcinogenesis was compatable with the age distribution of human tumors. According to that theory, a tumor would require seven specific mutations before becoming manifest.

a) Initiation: The first step of carcinogenesis was called initiation phase (Friedewald and Rous, 1944); in Berenblum's (1947) words this "implies the development of latent tumor cells as definite (macroscopically invisible) foci, from which visible tumors may ultimately develop". A large, single application of carcinogen did not behave as a sudden pulse stimulus (Berenblum et al., 1958), but may be retained sufficiently long to promote tumor grow. However, observations with susceptible mice requiring merely a few micrograms of carcinogen for tumor induction facilitate a sharp distinction between initiation and promotion (Boutwell, 1964). It is conceivable that carcinogenesis brings about an irreversible modification of the cell's DNA that eventually culminates in the formation of a cancer cell. Initiation occured only when a cell was in a particular metabolic state related to the mitotic cycle, involving critical metabolic changes that required at least one day for completion, and further, that actinomycin D specifically interfered with the particular metabolic pathway (Gelboin et al., 1965).

When DMBA was administered by stomach tube to partially hepatectomized mice at a time the regenerating liver cells were in peak period of DNA synthesis, a marked inhibition of DNA levels in the liver cells was not observed. But, when the DMBA was given during G_1, DNA synthesis was markedly inhibited (Raick, 1969). This implied that one of the actions of DMBA was not merely to affect the synthesis of DNA directly but to excert its effect also on the previous G_1 phase as well. This

effect may have been on such levels as, pooling of precursors or the regulation of their input to the DNA synthesis machinery.

Chivers et al. (1970) administered DMBA to mouse regenerating during S and G_2 phases. The rate of mitosis remained unaffected when DMBA was administered during the S phase, but the carcinogen accelerated the passage of cells into mitosis when they were treated while in G_2.

From a study of DMBA inducing mammary gland and uterine carcinogenesis in rats, King and Cowan (1970) concluded that DMBA inhibition of DNA synthesis was not directly related to its carcinogenic action.

Although the definitions of "initiator" and "promoter" are interdependent, an initiator acts rapidly and a single application is often effective. Attempts to block the carcinogenic action were generally unsuccessful (Tannenbaum, 1944), and the effects of the initiator were permanent (Berenblum and Shubik, 1947, 1949; Roe and Salaman, 1954). Although chemical carcinogens were usually considered as though they were distinctly separate agents, every initiator excerts some promoting activity and vice versa. The initiator is predominately an initiator while the promoter has its own predominate action (Salaman and Roe, 1964).

The essential activity of an initiator was demonstrated to be local, although tissues remote from the application site may occasionally be affected as well (Berenblum and Haran-Ghera, 1957). Some authors (Friedewald and Rous, 1950) suggested that initiators caused a mutation, producing a state of "latent neoplastic potentialities". Moreover, Salaman and Roe (1964) proposed the possibility that all cells were potentially capable of neoplastic behaviour but this may be

inhibited normally. Accordingly, an initiator could release a small number of cells from that inhibition thereby producing latent tumor cells.

The carcinogen may react with DNA both in vitro (Boyland and Green, 1962) as in vivo (Heidelberger and Davenport, 1961). It might affect some enzyme system concerned with they synthesis of DNA by deranging the supply of substrate, reducing the ATP level, deleting a particular protein; or else the carcinogen may not act on DNA at all (Ritchie, 1966). It could also alter components in the RNA synthesis mechanism or interfere with protein synthesis. Further Pitot and Heideburger (1963) suggested that a carcinogen could modify genetic repressors.

The number of tumors produced per mouse was reported to be a linear function of the area of skin exposed to the carcinogen and of the logarithm of the amount of carcinogen that entered the skin (Ball and McCarter, 1960; McCarter et al., 1956). Thus, McCarter (1966) observed that the absorption of small quantities of DMBA (0.05 µgm / cm^2) can initiate tumor yields in the same numerical range as those produced by much larger doses (up to 40 times larger).

Incorporation of H^3-T by cells of the mouse hair follicle was partially inhibited by application of DMBA (McCarter and Quastler, 1962a), and the duration of S was also prolonged (McCarter and Quastler, 1962b). McCarter (1966) asserted that BP could form stable complexes with DNA.

MCA, BP, and DMBA applied to mouse skin were observed to cause swelling of the epithelial cells and their nuclei, as well as vacuolation of the cytoplasm. Non-carcinogenic hydrocarbons did not produce such changes (Pullinger, 1940, 1941). The area of mouse skin exposed

to MCA exhibited a considerable variability in reaction (Cramer and Stowell, 1942).

Orr (1934, 1938) suggested that dermal fibrosis leading to ischemia might precipitate skin carcinogenesis. Other dermal changes following carcinogen administration were the derangement of the normally parallel bundles of collagen fibres to loosely distorted fibres, alteration in the elastic tissue composition and passive congestion of blood vessels. When MCA treated epidermis was transplanted to a normal site of autologous animals, tumors did not develop. When normal epidermis was transplanted to a site denuded of epithelium which previously had been treated with carcinogen, tumors arose as well (Billingham et al., 1951; Marchant and Orr, (1953). Orr (1963) concluded from these experiments that the essential changes in epidermal carcinogenesis occurred primarily in the dermis. This view met with some skepticism, however (Berenblum, 1959). Using whole thickness skin grafts, Salaman (1959) and Cowen (1959) were unable to confirm Orr's earlier observations. Mice which were untreated but carried carcinogen treated skin graft developed a few tumors on the host skin but many more on the grafted skin. The mice that were carcinogen treated and bore untreated skin grafts developed a large number of tumors on the host skin but few on the grafted skin. These observations did not permit a general conclusion that the state of initiation was necessarily a property inherent to the dermis.

If it is granted, however, that the immediate reaction of the carcinogen was to transform a few epithelial cells into latent tumor cells, the carcinogen could also penetrate and accumulate in the dermal fat. The dermis would then be capable to gradually release the carcinogen into the epidermis where inturn additional cells become transformed

(or affect the originally transformed epidermal cells). If this were the case, both sets of experiments described previously would not necessarily be contradictory.

Indeed Flesher and Sydner (1970) asserted that in mammary gland carcinogenesis with labelled DMBA, the level of carcinogen in the mammary fat was one of the critical determinants.

On the second day after a single application of MCA or DMBA in acetone to the back skin of male mice, Setala et al., (1959) noted that the IFE had become considerably thicker. The number of cells remained unaltered, but the cells themselves, and their nuclei, had become larger, the cytoplasm was vacuolated and the amount of keratohyaline granules became in instances augmented. After a single application of DMBA in acetone both Raick (1969, unpublished data) and Frei (1962) noted ultrastructually that the intercellular spaces were considerably widened. The normally tight junctions disappeared (Frei, 1969 pers. comm.) but the number of desmosomes and hemidesmosomes unchanged. Raick (1969, unpublished data) noted further that the cells which were not adjacent to the basal membrane, but still not yet fully differentiated apparently regained some metabolic activity, as it was evident from the reappearance of organelles in their cytoplasm. By the sixth day after carcinogen application Setala et al. (1959) observed that the IFE cells had become hyperplastic and hyperkeratotic. Mitosis was observed not only in the basal layer but also in the prickle cell layer.

Relative to the hair follicles, Setala et al. (1959) ascertained that by the second day after carcinogen application destructive changes had occurred, the sheaths were hyperplastic and the follicles contained

keratotic plugs. Between the sixth to tenth days the sebaceous glands disappeared almost entirely and many follicles were epilated.

A single application of MCA in benzene to the ears of hairless mice resulted in an increased level of cell loss from the epithelium after 5 days. It was followed by transient hyperplasia (Skjaeggestas, 1968).

Pullinger (1940) noted that 24 hours after a single application of one drop of o.3% MCA in acetone to the nape of the neck of mice aged between 6 - 8 weeks, there was considerable hypertrophy of the epidermis as well as an enlargement of the cell nuclei. The chromatin had collected on the nuclear border and surrounded the nucleoli. Two days after treatment there were not only more cells but both cells and nuclei were enlarged by 2 to 3 times. On the following day, numerous dead cells were observed but also many mitotic figures. Four days after the MCA treatment the size of the cells and nuclei began to decrease. Active cell division still persisted. By 10 days these acute changes occured only in a few local areas.

Applying a single considerably large dose of DMBA (0.75 mg.) in acetone to the skin of female BALB/of/Sp, March (MAf/Sp) and C_3H/Sp mice, Bond and Orr (1969) noted that all of the superficial layers of the epidermis, the distal parts of the hair follicles and sebaceous glands were destroyed by the fourth day. Eight days after treatment regeneration commenced, and hair follicles became differentiated de novo. Tumors arose after further treatment with croton oil from the superficial IFE at a later stage. This very large dose also destroyed oocytes, but did not produce necrosis in the adrenal gland.

It is evident from the variety of observations made of responses

induced by different concentrations of various carcinogens to different sexes and strains of mice, that not sufficient studies were yet conducted to elucidate the problem in an orerly and well controlled manner in longer ranged experimental series. Although sex, strain, dosage, and type of carcinogen are all significant in carcinogenesis, it remains still uncertain to what extent each one of these factors contribute to the process.

b) Promotion: Bullough and Laurence (1966) stated that promotion was the process whereby dormant cells (cells with latent neoplastic potentialities) were stimulated to multiply to a point at which they become identifyable tumor cells. A promoting agent was anything that provoked and accelerated this process. Relative to the action of initiators, the promoter tended to act more slowly and progressively than the carcinogen itself, requiring prolonged periods of treatment. The actions of promoters can be modified or inhibited, as by calorie restriction, for instance (Tannenbaum, 1944). Hyperplasia was frequently associated with tumor promotion but it may not be sufficient alone. Dammert (1961) suggested that epidermal hyperplasia was itself promoting only if it was supported by chronic inflamatory changes in the dermis. Van Duuren (1969) concluded that there was "no convincing evidence that hyperplasia or more extensive skin damage is related to the mechanism of action of tumor promoting agents". Low doses of promoter substances have been shown to be more effective than high doses.

If the concept is accepted that an initiator caused a state of latent neoplatic cells within a cell population, promotion would hasten it or determine its phenotypic expression (Berenblum, 1954). If the release from inhibition is the mechanism of the initiator then promotion

may again accelerate this release from the inhibitor.

Shear (1938) employed the term cocarcinogen to describe a noncarcinogenic substance that augmented the action of a carcinogen. Berenblum (1969) listed several possibilities by which a cocarcinogen could act. For instance, the cocarcinogen could alter the rate of diffusion of carcinogen from the site of administration to the target cell by acting as a solvent. Or, the cocarcinogen could alter the rate of the metabolic breakdown of the carcinogen. Finally, a cocarcinogen could affect the rate of elimination of the carcinogen, or of its active metabolite, from the tissue and body.

After applying phorbol ester of Croton Oil (Fraction A_1) to the back epidermis of female Swiss ICR mice, Raick (1969 pers. comm.) ascertained that the optimum dosage level was 0.016 μM of the fraction in acetone. This dose was capable of producing pronounced hyperplasia but did not cause other secondary changes, such as ulceration or severe necrosis of the IFE, which might likewise affect promotion. That author suggested further that the dose of the promotor was as critical as that of the "initiator" in two-stage carcinogenesis. Promoting doses applied at one week intervals were suggested, because the epidermal hyperplasia began to decline on the fifth day after painting, but still remained evident on the seventh day. The phorbol ester fraction of Croton Oil was determined to be at least 50 times more potent as a phlogistic and hyperplastic agent than the crude oil preparation. At all time intervals and dose levels that were studied hyperplasia was observed. In both, skin painted with crude oil and that with its fraction, vacuolation was evident of the cells lying at the dermo-epidermal junction and was most notable in the basal cell layer. This vacuolization became

apparent between 12 and 24 hours after painting, but not for the remaining part of the seven-day experiment. The fraction treated mice exhibited more focal areas of intense inflamatory infiltrate and necrosis of the epidermis.

After a single application of the A_1 fraction of Croton Oil in acetone to the back skin of 6 week old female Swiss ICR mice during Telogen, a variety of molar concentrations (0.16 μM, 0.016 μM, 0.0016 μM and 0.00016 μM) caused all hyperplasia of the IFE (measured as the thickness of the IFE) [Raick and Chivers, 1969 unpub. data]. The highest of these concentrations destroyed the entire epidermis by 96 hours, whereas 0.016 μM destroyed a great proportion of the epidermis but not all the cells. A concentration of 0.0016 μM produced an epidermal thickening up to 48 hours, but this declined again between 72 to 144 hours. Apart from the 0.16 μM concentration which killed all epidermal cells, hyperplasia essentially disappeared by 48 hours after the treatment.

As the prime concern of the present investigation was not so much the mechanism of the initiation or promotion of carcinogenesis, the literature review on this topic is accordingly selective and brief. A more extensive review of the problem of initiation and promotion was provided by the treatires of Frei (1962) and Shinozuka (1963).

CHAPTER III

METHODS AND MATERIALS

1. Test Animals

Male SWR mice (Manor Farms, Staatsburg, New York), 4 to 5 weeks old at the beginning of the experiment were used. Their average body weights on receipt was 25 grams and at the time of sacrifice 34 grams.

The animals received Victor Fox pellets and tap water ad libitum. Each animal was kept separate in either a metal or a plastic cage. Some of the plastic cages were fitted with metal dividers to separate each cage into two compartments housing one mouse each. The animals were maintained in a constant temperature and humidity environment, under controlled lighting conditions in a special environment chamber. The temperature was constant at $78^{\circ} \pm 2^{\circ}$ F., and the humidity was maintained at over 50% relative humidity. The daily cycles of illumination were from 8 a.m. to 8 p.m. artificial light, and darkness from 8 p.m. to 8 a.m.

2. Carcinogen

The compound employed as the carcinogenic agent in this investigation was 3,4 benzopyrene (L. Light and Co., Colnbrook, England and Koch-Light Laboratories Ltd., Inbrook, England).

3. Artificial Initiation of the Hair Growth Cycle

To initiate synchronous hair growth cycles, the hair was plucked with a pair of blunt forcepts, from an area of back skin at lease 2 cm. in diameter. The hair was thus removed from the skin in the midline, anterior to the pelvic girdle, at about the level of the lumbar vertebrae. The first artificially induced hair growth cycle was determined to be of approximately 19 days duration. The second hair growth

cycle was thus considered to commence 20 days after the hair had been plucked.

The first group of experiments consisted of two experiments which investigated the cytokinetics of Telogen and of Anagen VI IFE, that is the range of normal cytokinetic activity.

Sixteen days after plucking, a group of 48 mice were injected with tritiated thymidine at 10 a.m. and killed in groups of 4 mice at hourly intervals until 10 p.m. This group of animals remained otherwise untreated and served as the Anagen VI control group.

On day 20 after plucking, a second group of animals was injected with tritiated thymidine and were killed in a similar manner. This group of animals likewise received no treatment and served as the Telogen control group.

4. Administration of Tritiated Thymidine

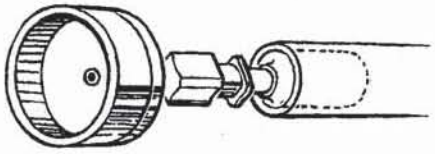
Each animal of a particular group to be sacrificed received a subcutaneous injection of 0.1 ml. aqueous solution of tritiated thymidine (25 μc per mouse) at 10 a.m. The injection site was caudal to the interscapular region but anterior to the plucked area. The tritiated thymidine was obtained from Nuclear-Chicago (Amersham-Searle), Des Plaines, Ill., as TRA-120 thymidine (methyl-T), s.a. 500-5000.

5. Carcinogen Application

The carcinogen benzopyrene was applied to the 2 cm. squ. area of plucked skin as 0.03 ml. of a 0.5% solution of benzopyrene dissolved in benzene. It was applied by means of a 2 ml. Repette Syringe fitted with an applicator especially constructed by Mr. Jones of the Science

Figure 6

Carcinogen Applicator



Technical Laboratory, The University of Manitoba. This particular applicator (see Fig. 6) consisted of a trucated 16 gauge needle soldered to a stainless steel plate backing the apparatus.

The second group of experiments consisted of six experiments, the first experiment at 20 days after the beginning of carcinogen treatment and each following experiment being twenty days after the previous one up until 120 days.

Each animal of the benzopyrene treated groups received a single such application each day (in the early afternoon) for ten consecutive days beginning with twenty-four days after plucking. This day was equivalent to the late phase of Anagen VI, the following days were Telogen phase. In the experiment designed to study the cytokinetics on the 20th day after benzopyrene application, no further administrations of benzopyrene were given after the original ten applications to the animals in this experiment. In the remaining experiments [IFE after 40, 60, 80, 100 and 120 days from the beginning of benzopyrene carcinogen treatment] BP was applied twice weekly beginning with four weeks after the first benzopyrene dose, and this secondary treatment was terminated approximately two weeks before the animals were killed.

The final group of experiments consisted of four experiments. The animals bearing tumors were withdrawn from the previous group (carcinogen treated animals) and classified as to tumor type after histological examination. The tumor bearing animals were killed after at least two weeks of no carcinogen treatment.

In conclusion, each benzopyrene treated animal received the carcinogen on ten consecutive days, this period spanning the Telogen stage. At the conclusion of the second hair cycle (that is, about four weeks after

the first administration of benzopyrene) the carcinogen was applied twice weekly until two weeks before the animal was killed.

6. Sacrifice and Histological Technique

The mice were killed by cervical neck dislocation at one hour intervals after the 10 a.m. injection of tritiated thymidine.

Tissue specimens from the plucked skin areas were fixed in Davidson's fixative. Routinely prepared 5 μ paraffin sections were prepared for radioautography and exposed for 2 to 5 days depending upon the emulsion used (NTB 1 or NTB 2) and the batch. After the slides were developed, fixed, and washed, the sections were stained with haematoxylin and eosin.

7. Enumeration of Cells

All sections were examined with a binocular light microscope under an oil immersion lens (magnification of 1000X). The numbers of labelled mitotic figures and also the total number of mitotic figures were determined among a population of at least 2000 interfollicular epidermal cells. This data was expressed as percentage labelled mitotic figures. As most tissue sections were about 2 cm. long, such sample population usually contained more than 2000 interfollicular epidermal cells.

The sections from animals administered with the radioactive label, tritiated thymidine, one hour prior to sacrifice, were also scored to determine the Labelling Index, that is, the number of labelled cells/ total number of cells.

The cells in sections of animals that were killed at 4 p.m. were scored to determine the percentage mitosis, that is, the number of mitotic figures per total number of cells. This was expressed as per cent mitosis.

A grid divided into 100 equal squares was placed in one eyepiece of a binocular microscope and the number of mitotic figures in the entire length of IFE was scored at 900X magnification. When estimating the nS, the number of labelled nuclei in the entire length of the IFE sample was determined. The number of basal cells in the IFE was then estimated for both MI and nS. The length (L) of the IFE strip was estimated under low power (i.e., 4X objective lens) in relative units of the micrometer disc. The mean number of basal cells per field (c's/F) were ascertained in the IFE with a 90X objective lens. The magnification factor (mF) was calculated as the ratio of the two objective lens

used (90/4 = 22.5). The number of cells in the IFE was calculated by multiplying.

$$\Sigma \text{ cells} = L \times C's/F \times mf \qquad (11)$$

The MI was then determined by

$$MI = \frac{100 \times \#\text{ of mitosis}}{\Sigma \text{ cells}} = \% \qquad (12)$$

and nS was calculated by

$$nS = \frac{100 \times \#\text{ of labelled cells}}{\Sigma \text{ cells}} = \% \qquad (13)$$

One modification was added when the MI and nS of squamous cell carcinomas were determined. The depth of the carcinoma was used instead of its length and as the tumor was solid, the number of cells per field was estimated by including nuclei that were a) entirely within the grid or b) touching the upper or the right side of the grid. Using these criteria, none of the cells were counted twice and none were theoretically missed.

8. Analysis of Labelled Mitotic Figures Curve

Each experimental series, composed of about fifty mice treated identically, was assumed for purposes of the first analysis to consist of 4 samples per hour covering 12 hour periods. That is, it was assumed that all samples were derived from a single animal. The data were processed by an IBM 360 computer of the Computer Department for Health Services of the University of Manitoba, and the curve of best fit was determined. This procedure was followed to determine the characteristics and pecularities of each curve, that is, whether the particular curve exhibited a quadratic function, a cubic function, a quartic function, or higher function.

In the second analysis, each of the four hourly estimations of percentage labelled mitoses was assigned, at random, to be part of either mouse 1, mouse 2, mouse 3, or mouse 4. That is, in the first analysis it was assumed that there was one mouse only with 4 samples per hour for twelve hours; the second analysis it was assumed that there were 4 mice with one sample per hour for twelve hours. These data were processed through the computer and the curve of best fit of the function determined from the first analysis. The values for the points at which the labelled mitotic figures curve crossed the 50% level were estimated. For instance, in the case of quadratic equations i.e.

$$f(x) = ax^2 + bx + c \tag{14}$$

these values were calculated by the formula for finding the roots of a quadratic equation:

$$x = \frac{-b \pm \sqrt{b^2 - 4ad}}{2a} \tag{15}$$

where $d = c - 50$.

The time from zero to the lower root was equivalent to the duration of $(1/2\ M + G_2)$ and the difference of the roots was equivalent to the duration of

$$x_2 - x_1 = tS \tag{16}$$

where x_2 is the greater root, x_1 is the smaller root and tS is the duration of the DNA synthesis phase.

The estimated quadratic functions of the curve were also plotted and the roots at the 50% values were estimated visually. The two estimates, calculated and graphical agreed to the first decimal place.

In the case of functions of a power higher than a quadratic function, the estimated values of the functions were accurately plotted

and the roots at the 50% values were estimated by visual inspection, at an accuracy of 1/10th of an hour.

9. Estimation of Generation Times

The generation time was estimated in experiment 4 by ascertaining the percentage of labelled basal cells/labelled cells for 5 days following tritiated thymidine administration. The point at which the curve fell below 50% yielded an estimate of the generation time (tC).

In all experiments the method of Johnson et al., (1960) was followed to estimate the generation time. It is calculated by the formula:

$$tC = \frac{tS\ (\ln 2)}{\text{labelled cells/total cells}} \qquad (17)$$

where ln 2 = 0.69315

tS = duration of DNA synthesis

$$\text{labelled cells/total cells} = \frac{\text{Labelling Index}}{100} = \frac{nS}{100}$$

10. Estimation of the Mitotic Duration

The time required to complete the mitotic process (mitotic duration) was estimated by the formula:

$$tM = \frac{6MI}{MR} \qquad (18)$$

where tM = mitotic duration

MI = mitotic index = % dividing cells

MR = mitotic rate = % dividing cells per 6 hours

11. Estimation of G_2

Although a value for 1/2 M + G_2 can be derived from the labelled

mitosis curve, it constitutes a poor estimate. The reason is that it is not 1/2 M but merely a fraction of mitotic duration which is contained within this compartment. For calculation purposes only, it is conventionally assumed to correspond to 1/2 M. The duration of G_2 is estimated by the formula:

$$tG_2 = t\ (1/2\ M + G_2) - \frac{tM'}{2} \qquad (19)$$

where tM' derived from formula (5).

12. Estimation of G_1

All of the other components of the cell cycle being known G_1 is ascertained by mere subtraction of the other phases:

$$tG_1 = tC - (tM + tG_2 + tS) \qquad (20)$$

13. Statistical Methods

The values of the arithmetic mean, standard deviations, and standard errors, as well as tests of significance, but also the students t test and analysis of variance were determined from a standard statistical program at the University of Manitoba's Institute for Computer Studies. The formulae used to check some of the data were:

$$\bar{x} = \Sigma x/n \qquad \text{mean} \qquad (21)$$

$$\sigma = \sqrt{(\Sigma x^2/n) - \bar{x}^2} \qquad \text{standard deviation} \qquad (22)$$

$$S.E. = \frac{\sigma}{N} \qquad \text{standard error} \qquad (23)$$

$$t = \sqrt{\frac{(n_1 - 1)\ S_1^2 + (n_2 - 1)\ S_2^2}{n_1 - n_2 - 2}} \left[\frac{1}{n_1} + \frac{1}{n_2} \right] \text{ t value} \qquad (24)$$

and the analysis of variance (Speigle, 1961).

A 5% level of significance served as the standard to accept or reject the null hypothesis, unless otherwise stated.

14. Histological Area Investigated

Although one of the more intriguing areas of the present problem was the follicular ostium or peri follicular epithelium because of its association with benzopyrene induced squamous cell carcinomas (Fig. 5), this area was not the prime area of epithelium investigated. Part of the reason for not following this promising lead is the particular difficulty in defining exactly the follicular ostium. Also in the present study insufficient suitable samples were encountered to allow an adequate estimate of the Theoretical Labbelled Mitoses Curve of the Follicular Ostium.

Thus, for these reasons, the InterFollicular Epidermis was investigated, as well as the reason that the IFE was involved with both the histogenesis of both the malignant and benign benzopyrene induced tumors of the mouse.

CHAPTER IV

RESULTS

Results

In each of the twelve experiments reported, the results are presented in three sets of data: to begin with, the Experimental Labelled Mitoses Curve is presented. This table contains data of the means and standard errors, as well as the sample number of each experimentally determined value, and of the percentage of labelled mitoses. It is followed by the graph of the theoretical function which was derived from the first set of data. Finally, a resumé is presented of the growth parameters of the particular population that was investigated.

After all the experimental data are reported, the different stages of the cell cycle and mitotic indices are listed in tables of comparisons. The final table (34) presents the functions of the estimated theoretical curves and also the results of the analysis of variance.

1. Untreated Interfollicular Epidermis (IFE)

The IFE was studied in two stages of the hair growth cycle serving "normal" or control base lines. Telogen, or the resting stage, (Tables 2 and 3, Fig. 7) and Anagen VI, or the growing stage of the hair cycle (Tables 4 and 5, Fig. 8) were remarkably different during some aspects of their growth patterns. As the method used to ascertain tC and tG_1 was relatively unreliable and resulted in considerable variability, these values cannot be considered as being different on a statistical basis. However, the durations of S and G_2, and the fraction of cells synthesizing DNA (nS), were all significantly different in both physiological states ($p = 1\%$). In the Telogen IFE, a greater number of cells were synthesizing DNA ($3.06 \pm 0.13\%$) during equivalent periods, and they spent a longer time in the S phase (8.35 ± 0.24 hours)

A Further Note on the Development of the Data

In the development of the data, several steps were taken and these are summarized below:

1. Experimental data were listed and plotted (Appendix).
2. Curve of best fit of the theoretical curve of labelled mitoses was estimated (Table 34) and plotted (Tables 2 to 24, even numbers).
3. $t(1/2\ M + G_2)$ and tS were estimated from the theoretical curve.
4. Ml and nS were estimated by cell counts.
5. tM was calculated from $tM = \frac{6Ml}{MR}$.
6. tG_2 was calculated from $tG_2 = t(1/2\ M + G_2) - 1/2\ tM$.
7. Generation times were calculated $tC = \frac{tS\ ln\ 2}{nS}$.
8. tG_1 was calculated from $tG_1 = tC - [tS + tG_2 + tM]$.

Table 2

Experimental Labelled Mitoses Curve of Telogen IFE

Hours after H^3-T	N	Percent labelled mitoses
1	4	0.00 ± 0.00
2	3	5.67 ± 5.67
3	2	60.00 ± 35.00
4	4	76.50 ± 5.72
5	4	90.25 ± 6.84
6	3	84.00 ± 4.93
7	4	93.25 ± 4.15
8	4	96.00 ± 4.00
9	3	97.67 ± 2.33
10	4	80.50 ± 10.69
11	4	73.25 ± 3.61
12	4	26.50 ± 10.44

Figure 7

Labelled Mitoses Curve at Telogen

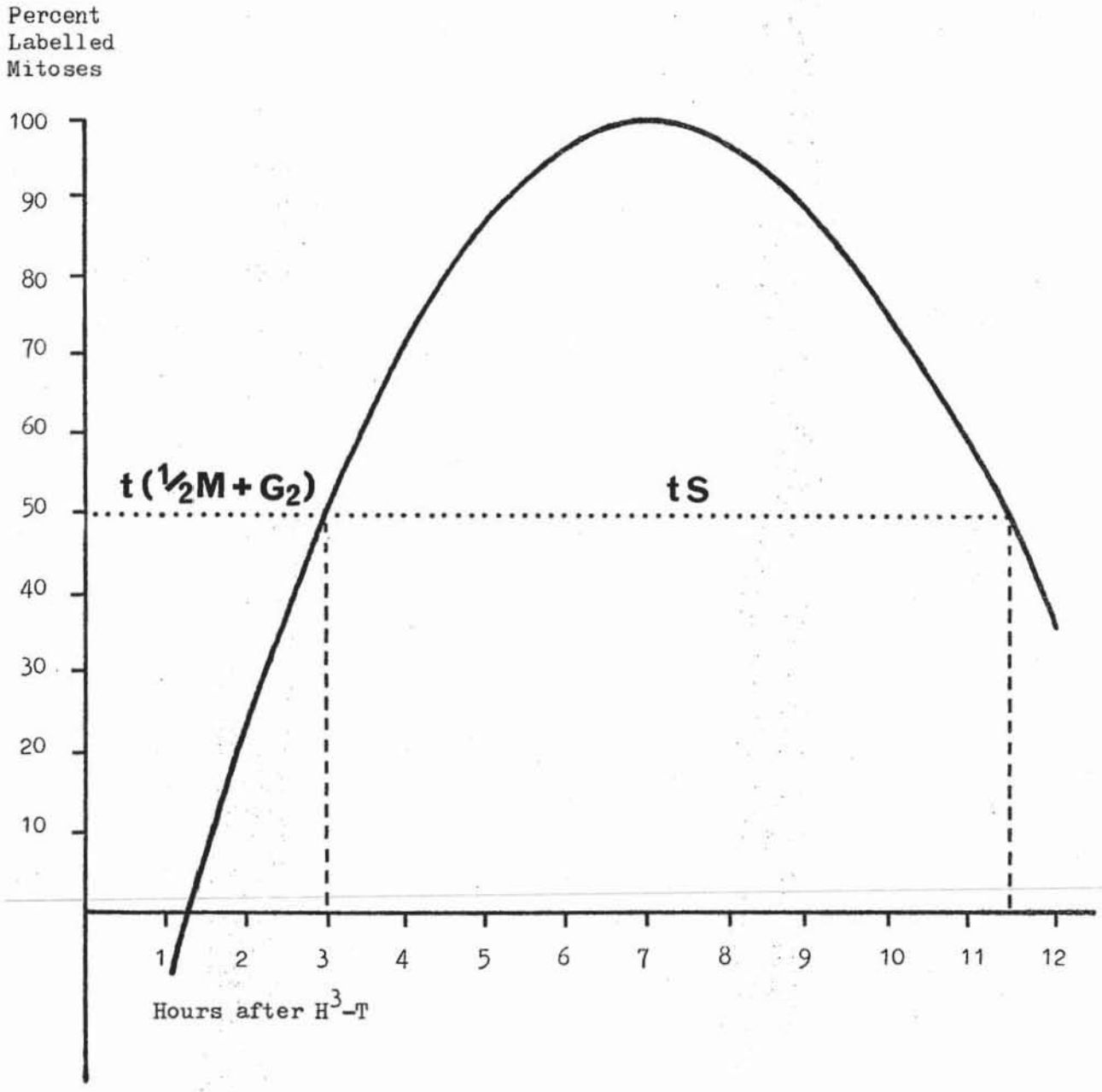


Table 3

Cytokinetics of Telogen IFE

tC = Generation Time	174.65 ± 6.62 hours
tG_1 = Duration of G_1	161.82 ± 6.81 hours
tS = Duration of S	8.35 ± 0.24 hours
tG_2 = Duration of G_2	2.37 ± 0.10 hours
tM = Duration of Mitosis	1.58 ± 0.31 hours
MI = Mitotic Index	0.19 ± 0.04 %
nS = Labelling Index	3.06 ± 0.13 %

Table 4

Experiemntal Labelled Mitoses Curve of Anagen VI IFE

Hours after H^3-T	N	Percent labelled mitoses
1	4	0.00 ± 0.00
2	4	17.00 ± 10.02
3	1	29.00
4	4	32.00 ± 4.53
5	4	73.00 ± 9.11
6	4	65.75 ± 8.38
7	3	54.00 ± 10.54
8	3	71.33 ± 14.89
9	3	58.33 ± 8.33
10	2	25.00 ± 25.00
11	3	0.00 ± 0.00

Figure 8

Labelled Mitoses Curve at Anagen VI

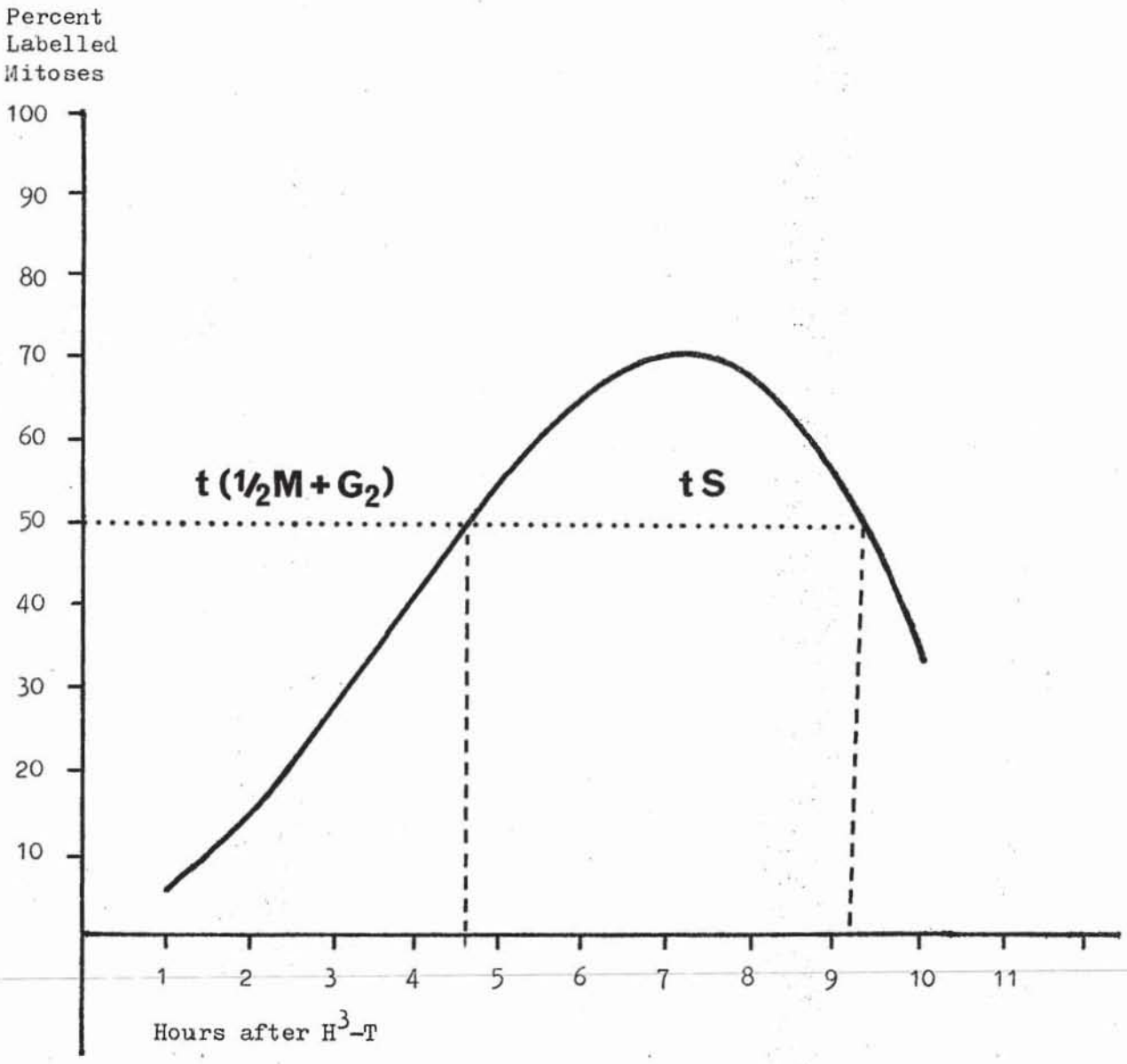


Table 5

Cytokinetics of Anagen VI IFE

tC	= 255.00 ± 53.17 hours
tG_1	= 246.00 ± 52.90 hours
tS	= 4.77 ± 0.22 hours
tG_2	= 3.74 ± 0.16 hours
tM	= 1.89 ± 0.43 hours
MI	= 0.31 ± 0.06 %
nS	= 1.20 ± 0.24 %

* Legend see Table 3.

but less time in G_2 (2.39 ± 0.10 hours). In Anagen VI IFE, fewer cells entered the S phase (1.20 ± 0.24%), these cells tarry in S phase for a brief period (4.77 ± 0.22 hours), but once they entered into G_2 they abided in that phase for a longer period (3.73 ± 0.16 hours). By the analysis of variance test, neither the duration of mitosis nor the mitotic index (MI) proved different in the untreated IFE on both physiological conditions.

The pooled values of the control data without significant variation were tC = 214.95 ± 29.11 hours, tG_1 = 203.86 ± 29.35 hours, tM = 1.74 ± 0.24 hours and MI = 0.25 ± 0.04%.

2. Carcinogen Treated Interfollicular Epidermis (IFE)

Six experimental series are reported, each carried through 20 days after the previous one, beginning 20 days after the initiation of carcinogen treatment. All experiments were begun in a similar manner, that is, by 10 consecutive daily applications of Benzopyrene (BP) during Telogen. The total initiating dose was 1.5 mgm. BP. Three weeks after the beginning of the daily carcinogen treatment, the applications of carcinogen changed to beweekly. Thus the animals of the first experiment received only the initiating dose, and were killed after 10 days without further treatment. In each succeeding experiment, six separate doses of 0.9 mgm. BP was applied in addition, during the following three weeks (Table 26).

When an animal was killed, the number of surviving animals bearing tumors was recorded. The carcinogenic index (percentage of surviving animals bearing tumors) versus time are reported in Figure 20. Although a curve was not fitted to this set of data, it appears to behave similar

to an exponental function during the test period.

The tables (6 to 16, even numbered) of experimentally labelled mitoses curves, the figures of the theoretical labelled mitoses curves (Figures 9 and 10, 12 to 15), and the duration of phases of the cell cycle (Tables 7 to 17, odd numbers) of the IFE treated at 20 day intervals with BP are presented. The ratio of labelled basal cells over the total number of labelled cells at 40 days after the commencement of carcinogen treatment (Fig.11) was determined to verify whether the calculated tC was plausible. The tC estimated by the ratio method was 128 hours, as compared to the calculated tC of 119.7 ± 40.0 hours; this signified that both methods for determining tC agreed roughly. The calculation method was chosen as the procedure of choice, as it required fewer animals and the estimate of variance was obtained more readily.

The date of the treated IFE were subjected to analysis of variance independently from the control IFE or tumor data. The following parameters displayed significant variability: tS ($p = 0.5\%$), tG_2 ($p = 5\%$), tM ($p = 1\%$), nS ($p = 0.5\%$), as well as the mitotic index ($p = 1\%$). The phases of the cell cycle that did not exhibit significant variability were tC, tG_1 and t (M/2 + G2) [Tables 27 to 33].

tS of Treated IFE

The students' 't' test revealed that the duration of DNA synthesis on days 20 and 40 was longer than that on days 80 or 100 ($p = 1\%$). Moreover, tS of day 40 was longer than tS of day 60 ($p= 5\%$). On day 120 the tS was larger than tS on day 60 ($p= 5\%$), day 80 or 100 ($p= 1\%$). Further, tS on days 20, 40 and 120 were not statistically different from each other. Also, tS of days 60 and 80 were similar.

tG_2 of Treated IFE

The duration of G_2 of IFE, 60 days after commencement of benzopyrene application, was longer than tG_2 on days 40 or 100 (p= 5%). tG_2 of day 80 was greater than the tG_2 of day 100 (p= 5%), and tG_2 of day 120 was still longer than tG_2 of both days 100 (p= 1%) or 40 (p= 5%). All other comparisons did not have statistically significant differences.

tM of Treated IFE

The durations of mitosis of treated IFE at days 40 and 60 were not different between themselves, although they both were longer than the tM of days 20, 80 (p= 5%), 100, and 120 (p= 1%). Other comparisons were not significantly different by the students 't' test.

nS of Treated IFE

The fraction of cells synthesizing DNA on day 40 after the beginning of carcinogen treatment was greater than the nS of all other treated cell populations (p= 1%). Other significant variation did not occur between the latter groups.

MI of Treated IFE

The mitotic index of the treated IFE at 40 days was higher than the MI at day 100 (p= 5%). MI at day 60 was larger than the MI at day 40 (p= 5%), 80, 100 and 120 (p= 1%).

tC and tG_1 of Treated IFE

The pooled values of treated IFE cell compartments which did not exhibit any statistically significant variability were: tC = 192.05 ± 16.96 hours; tG_1 = 182.87 ± 16.91 hours.

Table 6

Experimental Labelled Mitoses Curve of IFE 20 Days After Beginning of Carcinogen Treatment

Hours after H^3-T	N	Percent labelled mitoses
1	4	0.00 ± 0.00
2	4	15.75 ± 10.23
3	4	48.50 ± 13.11
4	4	75.75 ± 5.86
5	3	75.33 ± 2.60
6	4	70.25 ± 7.12
7	4	76.75 ± 10.70
8	4	79.25 ± 10.55
9	4	54.50 ± 16.05
10	3	29.33 ± 18.33
11	4	32.25 ± 6.33
12	3	10.33 ± 10.33

Figure 9

Labelled Mitoses Curve 20 Days After

Beginning of Carcinogen Treatment

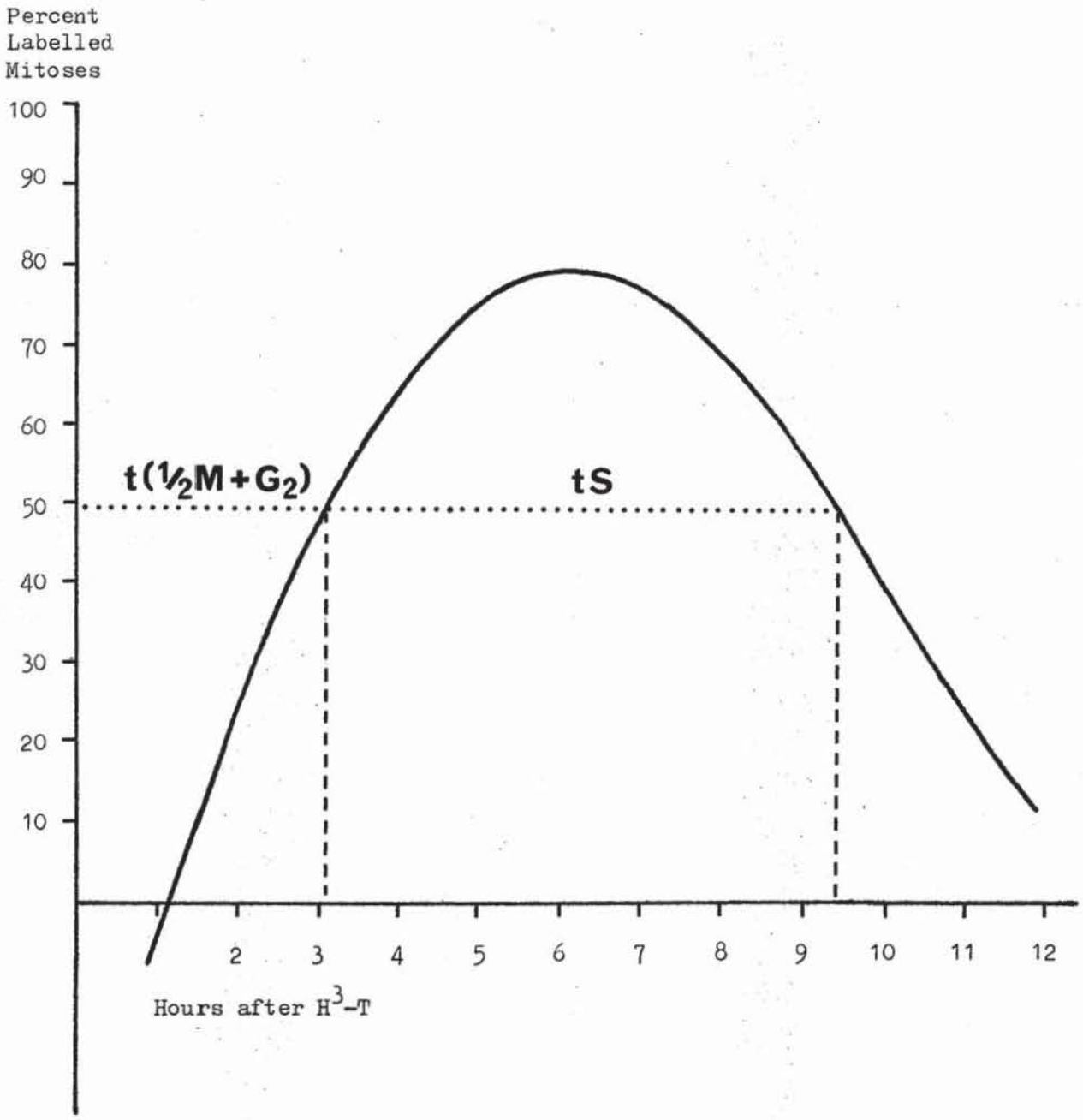


Table 7

Cytokinetics of IFE 20 Days After Beginning of Carcinogen Treatment

tC	= 187.50 ± 29.80 hours
tG_1	= 177.60 ± 30.00 hours
tS	= 6.35 ± 0.25 hours
tG_2	= 2.95 ± 0.43 hours
tM	= 0.59 ± 0.11 hours
MI	= 0.29 ± 0.01 %
nS	= 2.46 ± 0.25 %

* Legend see Table 3.

Table 8

Experimental Labelled Mitoses Curve of IFE 40 Days After Beginning of Carcinogen Treatment

Hours after H^3-T	N	Percent labelled mitoses
1	4	0.00 ± 0.00
2	4	9.5 ± 3.23
3	4	35.00 ± 4.04
4	4	60.50 ± 9.70
5	3	62.33 ± 7.54
6	2	70.00 ± 17.00
7	4	95.00 ± 5.00
8	2	94.50 ± 5.50
9	4	52.25 ± 10.66
10	4	38.50 ± 11.66
11	2	44.00 ± 0.00
12	4	9.25 ± 3.15

Figure 10

Labelled Mitoses Curve 40 Days After

Beginning of Carcinogen Treatment

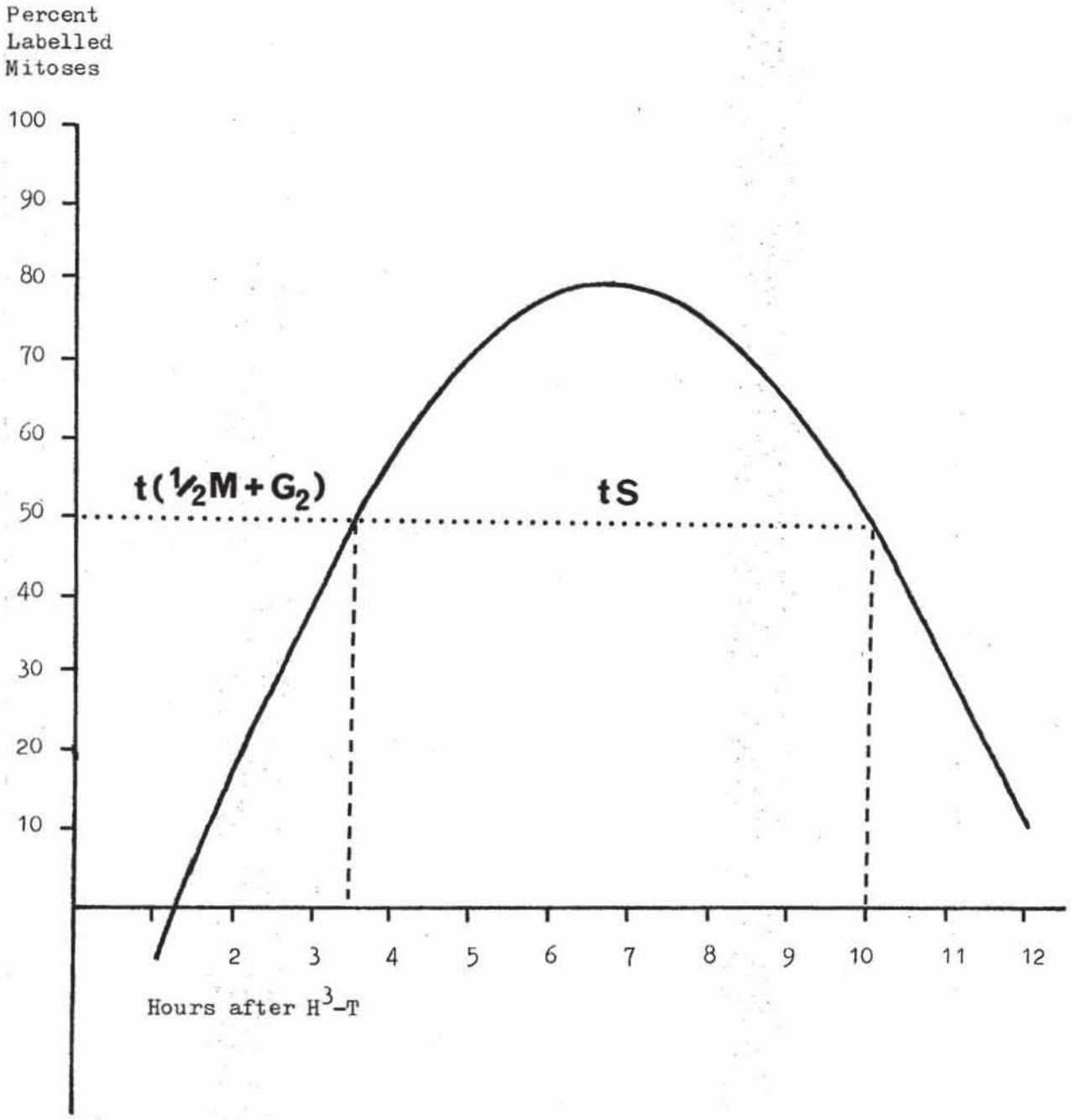


Figure 11

Ratio of Labelled Basal Cells : Labelled Cells

40 Days After Beginning of Carcinogen Treatment

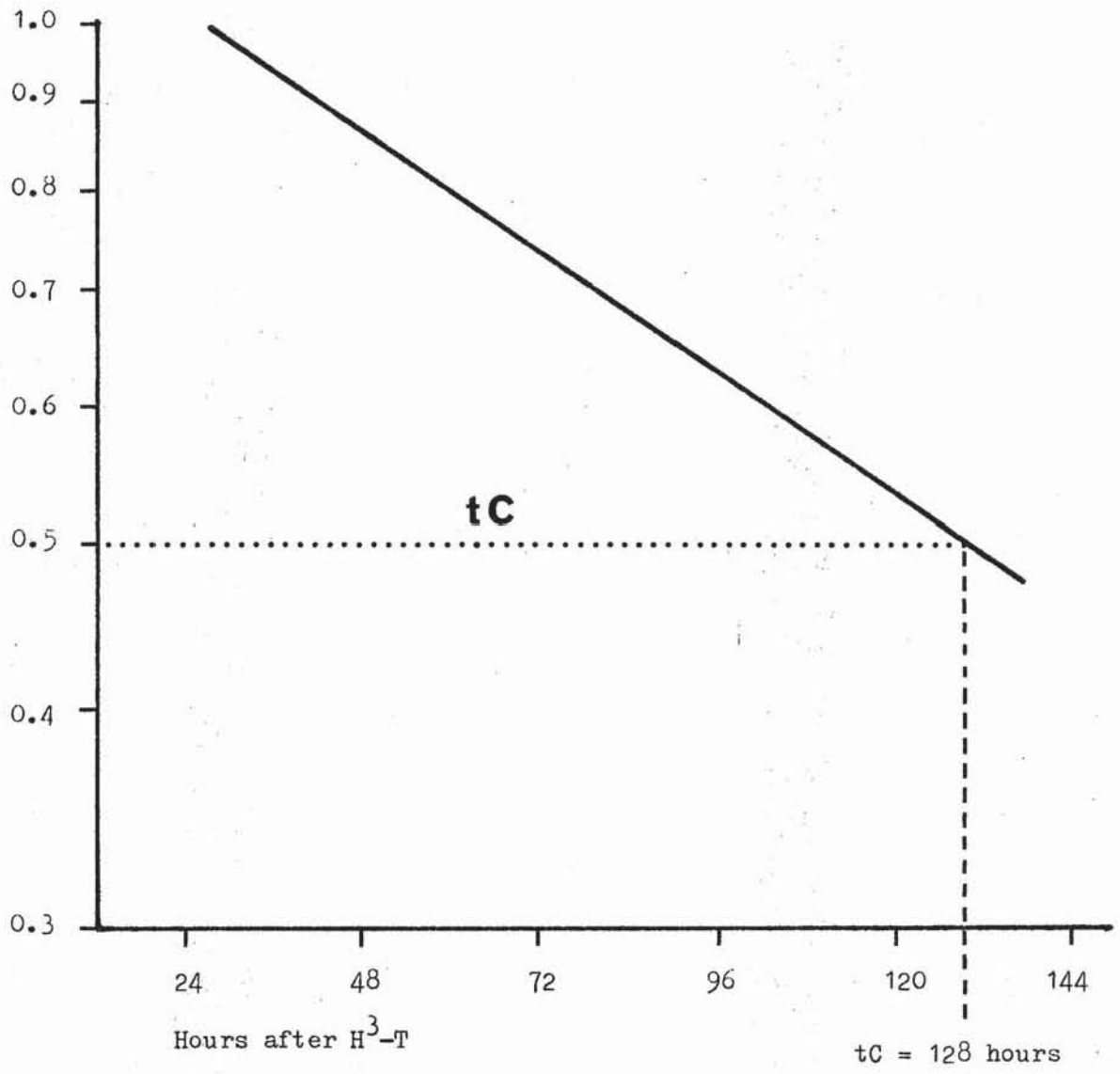


Table 9

Cytokinetics of IFE 40 Days After Beginning of Carcinogen Treatment

tC	= 119.7	± 44.0	hours
tG_1	= 109.1	± 44.0	hours
tS	= 6.72	± 0.34	hours
tG_2	= 2.71	± 0.33	hours
tM	= 1.55	± 0.38	hours
MI	= 0.35	± 0.06	%
nS	= 7.67	± 1.78	%

* Legend see Table 3.

Table 10

Experimental Labelled Mitoses Curve of IFE 60 Days After Beginning of Carcinogen Treatment

Hours after H^3-T	N	Percent labelled mitoses
1	3	0.00 ± 0.00
2	2	0.00 ± 0.00
2.5	2	39.50 ± 8.50
3	4	30.75 ± 8.79
3.5	2	39.75 ± 2.25
4	3	54.55 ± 17.50
5	4	51.25 ± 11.45
6	4	81.00 ± 6.43
7	4	75.63 ± 10.58
8	4	40.37 ± 11.11
8.5	4	59.50 ± 15.42
9	4	68.75 ± 10.84
9.5	4	25.55 ± 6.30
10	4	32.78 ± 7.16
11	2	6.25 ± 6.25
12	2	0.00 ± 0.00

Figure 12

Labelled Mitoses Curve 60 Days After

Beginning of Carcinogen Treatment

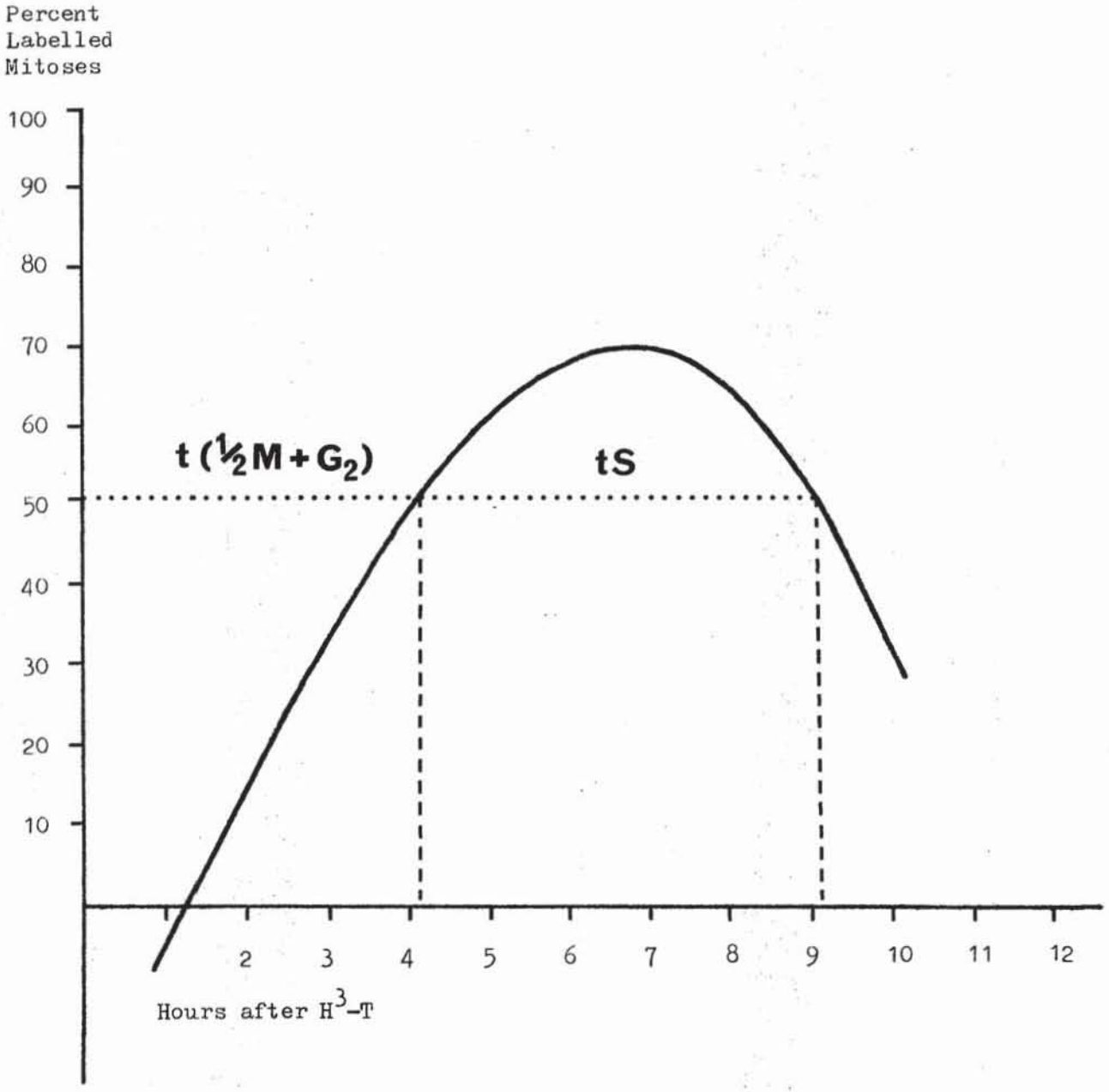


Table 11

Cytokinetics of IFE 60 Days After Beginning of Carcinogen Treatment

tC	= 210.7	± 77.4	hours
tG_1	= 199.9	± 80.0	hours
tS	= 5.33	± 0.27	hours
tG_2	= 3.87	± 0.15	hours
tM	= 1.35	± 0.33	hours
MI	= 0.51	± 0.08	%
nS	= 1.76	± 0.59	%

* Legend see Table 3.

Table 12

Experimental Labelled Mitoses Curve of IFE 80 Days After Beginning of Carcinogen Treatment

Hours after H^3-T	N	Percent labelled mitoses
1	4	0.00 ± 0.00
2	4	10.75 ± 7.78
3	4	24.00 ± 11.01
4	4	38.75 ± 5.99
5	4	62.50 ± 5.80
6	3	71.33 ± 3.18
7	5	75.25 ± 7.41
8	3	93.00 ± 7.00
9	4	47.75 ± 5.47
10	4	28.50 ± 7.86
11	1	5.00

Figure 13

Labelled Mitoses Curve 80 Days After

Beginning of Carcinogen Treatment

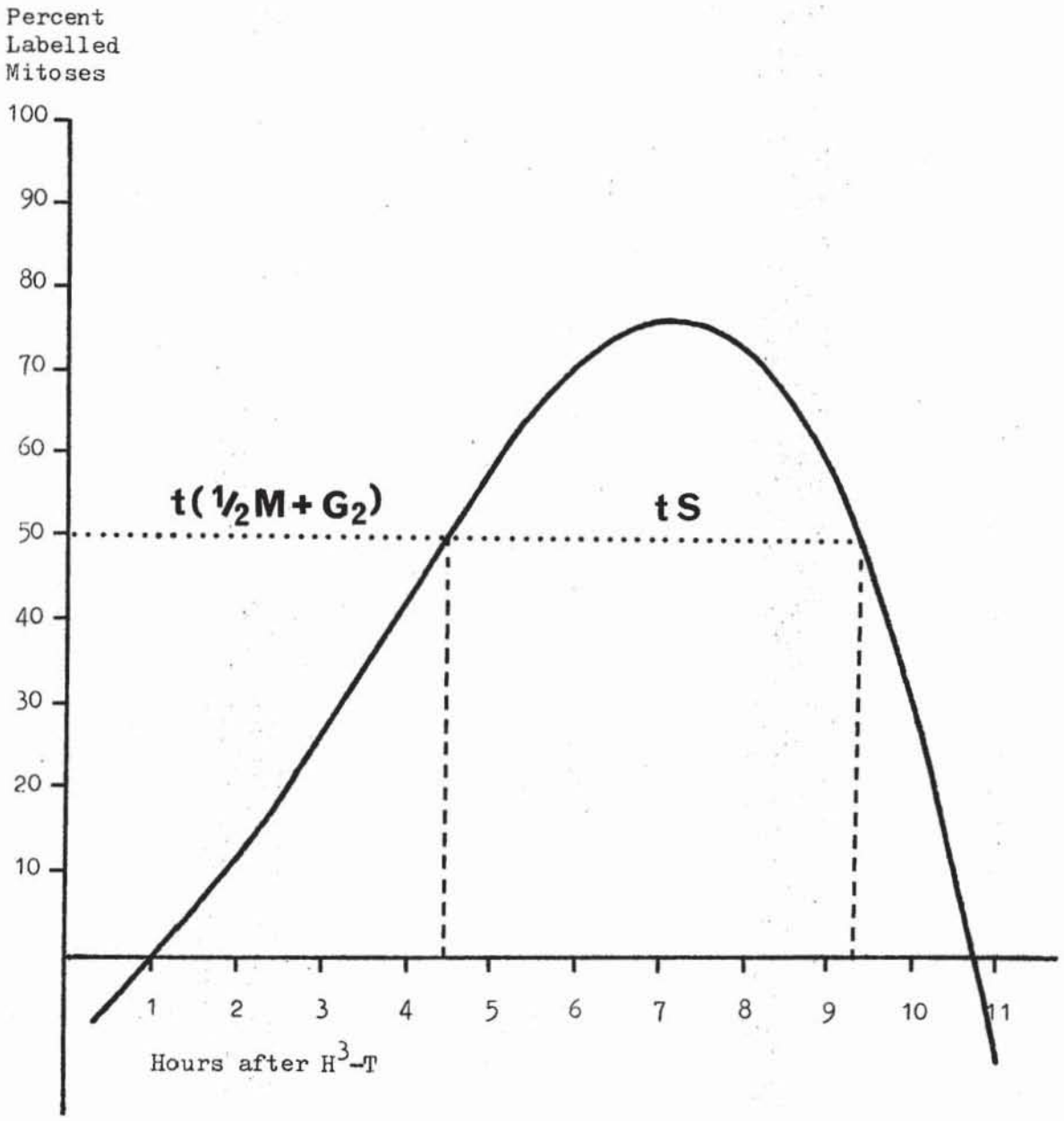


Table 13

Cytokinetics of IFE 80 Days After Beginning of Carcinogen Treatment

tC	= 196.7	± 24.7	hours
tG_1	= 187.6	± 24.2	hours
tS	= 4.65	± 0.31	hours
tG_2	= 3.58	± 0.18	hours
tM	= 0.59	± 0.25	hours
MI	= 0.21	± 0.09	%
nS	= 1.45	± 0.39	%

* Legend see Table 3.

Table 14

Experimental Labelled Mitoses Curve of IFE 100 Days After Beginning of Carcinogen Treatment

Hours after H^3-T	N	Percent labelled mitoses
1	4	0.00 ± 0.00
2	4	18.85 ± 8.30
3	4	31.25 ± 3.61
4	3	46.00 ± 13.53
5	4	80.25 ± 4.87
6	4	72.25 ± 5.25
7	4	58.25 ± 7.66
8	4	54.00 ± 16.67
9	4	22.87 ± 9.93

Figure 14

Labelled Mitoses Curve 100 Days After

Beginning of Carcinogen Treatment

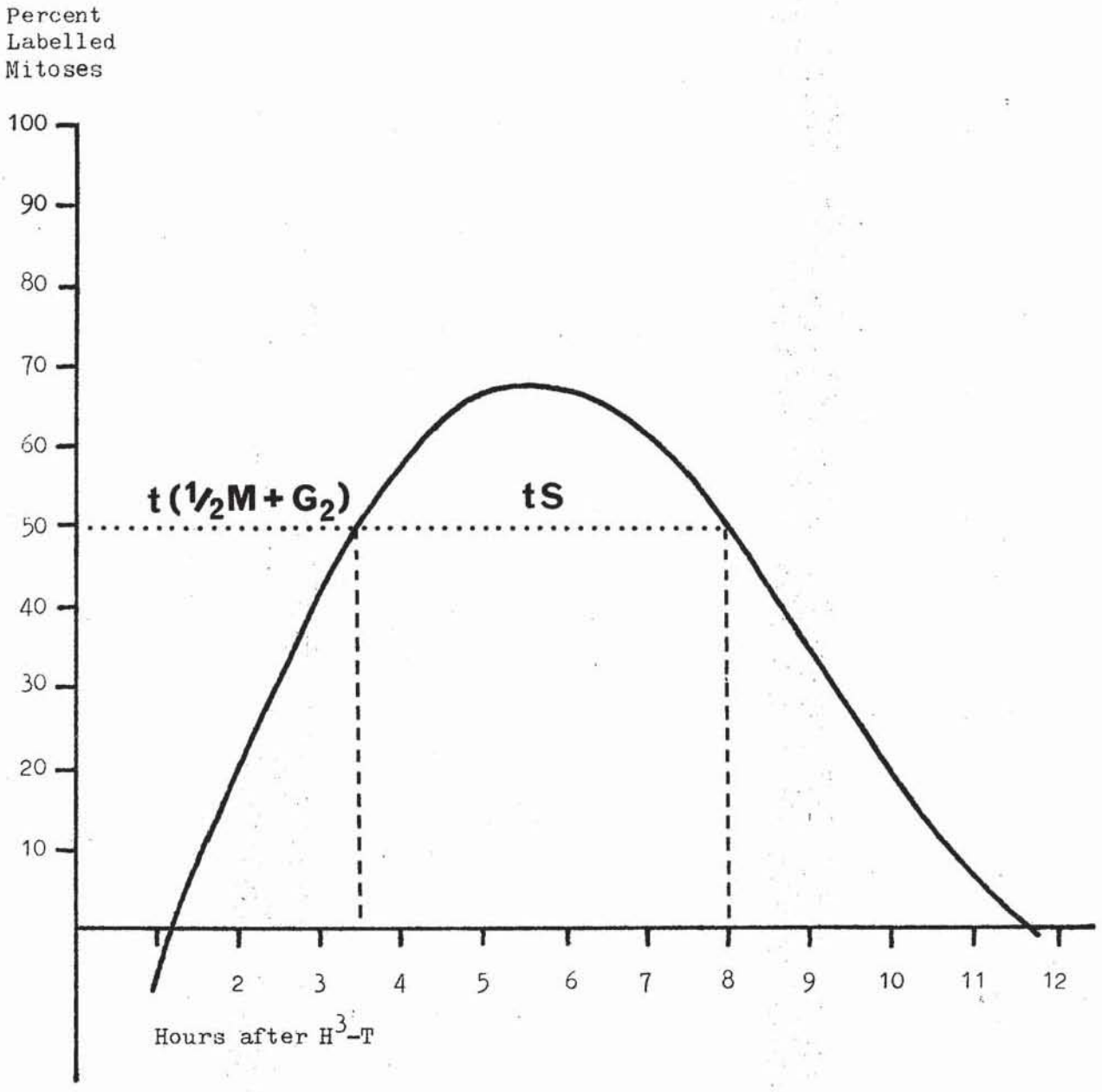


Table 15

Cytokinetics of IFE 100 Days After Beginning of Carcinogen Treatment

tC	= 234.2	± 49.8	hours
tG_1	= 222.5	± 45.6	hours
tS	= 4.47	± 0.58	hours
tG_2	= 2.35	± 0.40	hours
tM	= 0.15	± 0.03	hours
MI	= 0.19	± 0.03	%
nS	= 1.35	± 0.32	%

Table 16

Experimental Labelled Mitoses Curve of IFE 120 Days After Beginning of Carcinogen Treatment

Hours after H^3-T	N	Percent labelled mitoses
1	4	0.00 ± 0.00
2	4	9.25 ± 3.40
3	4	22.00 ± 9.83
4	2	45.50 ± 34.50
5	4	72.75 ± 10.45
6	3	65.33 ± 8.67
7	4	84.50 ± 6.20
8	4	69.50 ± 2.18
9	4	68.00 ± 4.88
10	3	54.67 ± 14.19
11	2	40.00 ± 0.00
12	4	30.50 ± 7.58

Figure 15

Labelled Mitoses Curve 120 Days After

Beginning of Carcinogen Treatment

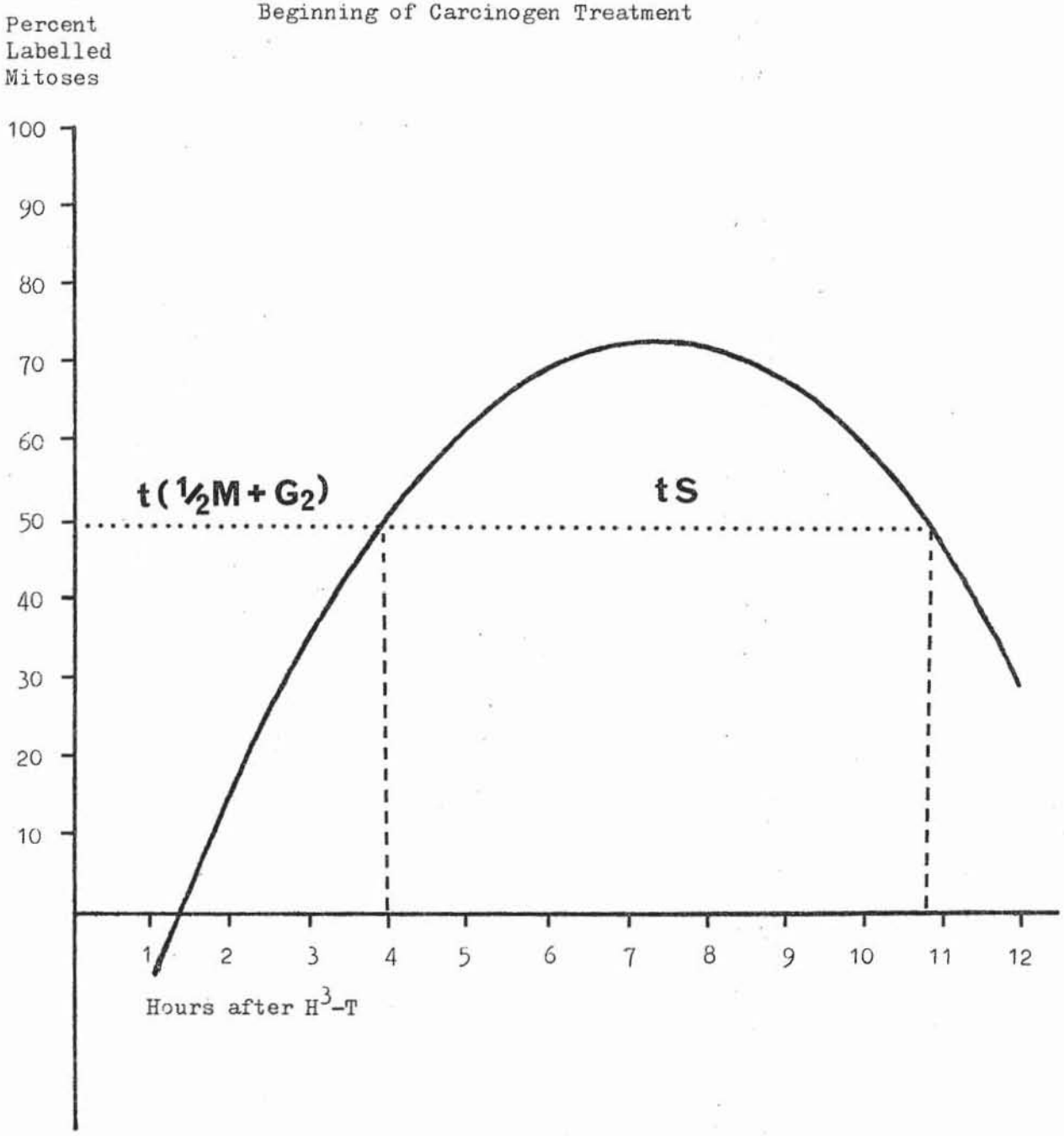


Table 17

Cytokinetics of IFE 120 Days After Beginning of Carcinogen Treatment

tC	= 203.4	± 27.3	hours
tG_1	= 197.4	± 28.9	hours
tS	= 6.90	± 0.44	hours
tG_2	= 3.92	± 0.44	hours
tM	= 0.44	± 0.11	hours
MI	= 0.19	± 0.01	%
nS	= 2.58	± 0.58	%

* Legend see Table 3.

3. Induced Tumors

When an animal was observed having developed a tumor, that animal was separated from the group of other treated animals. None of the tumor bearing animals were killed until they had been withdrawn from the biweekly benzopyrene treatment for at least two weeks. Several experimental series of tumor induction were performed; all the animals in these series were killed by 120 days after the beginning of treatment. After histological examination, each tumor was identified as to its type (EKA, MKA, RKA or SCC), and the percentage labelled mitoses was determined in suitable samples (Tables 18, 20, 22 and 24). The mitotic and labelling indices were estimated in those tumors of animals which had been killed at 4 p.m. and 11 a.m., respectively. As the EKA were particularly small, they were frequently noted first only during microscopic examination of the sections of treated skin. Many EKA-bearing animals derived from the treated groups, while others were from animals which frequently bore multiple tumors. The different histopathological stages of development of the deratoacanthoma are presented individually, EKA (Fig. 16, Tables 18 and 19), MKA (Fig. 17, Tables 20 and 21) and RKA (Fig. 18, Tables 22 and 23). The final experimental data are those of the cytokinetics of the benzopyrene induced squamous cell carcinoma (Fig. 19, Tables 24 and 25).

Analysis of variance indicated that the following parameters displayed significant variability between tumors: tG_2 ($p = 0.5\%$); tM ($p = 5\%$); and mitotic index ($p = 1\%$).

Tumor tC, tG_1 and tS

The analysis of variance test indicated that significant differences did not exist between the generation time, the duration of G_1,

or duration of DNA synthesis in the four tumor types under study. The pooled values for these parameters were tC = 32.39 ± 4.58 hours; tG_1 = 22.93 ± 4.61 hours, and tS = 5.68 ± 0.11 hours.

Tumor tG_2

The duration of G_2 of the RKA (1.49 ± 0.07 hours) was shorter than tG_2 of the other tumors (p= 1%). Other differences were not significantly statistically, however.

Tumor tM

The duration of mitosis of the Regressing Keratoacanthoma (2.94 ± 0.38 hours) was longer than the tM of the other tumors, EKA (0.35 ± 0.07 hours) [p= 1%], MKA (1.26 ± 0.08 hours), or SCC (1.32 ± 0.80 hours) [p= 5%]. Other differences were not apparent.

Tumor nS

The analysis of variance did not reveal any variability between the DNA synthesis fractions of tumor cells. The pooled nS for all tumors investigated was 15.32 ± 1.74%.

Tumor MI

The mitotic index of the benign tumors was greater than the MI of the squamous cell carcinoma (p= 1%). The MI of the RKA was higher than that of the MKA (p= 1%), while the MI of the EKA was not different from that of the MKA or the RKA.

4. Comparison Between the Cell Cycle Phases

In this section each parameter is compared within itself to all conditions studied. The comparisons within a group are reported elsewhere, such as between untreated Telogen and Anagen VI; these "control" data are presented in the section on the untreated IFE. Such comparisons

Table 18

Experimental Labelled Mitoses Curve of

Early Keratoacanthoma

Hours after H^3-T	N	Percent labelled mitoses
1	4	0.00 ± 0.00
2	1	0.00
2.5	3	20.00 ± 11.55
3	1	90.00
3.5	1	60.00
4	4	83.25 ± 4.23
5	4	87.25 ± 2.45
6	4	66.25 ± 10.18
7	4	58.25 ± 9.37
8	4	61.00 ± 13.55
9	4	36.00 ± 11.78
11	4	20.75 ± 6.74
12	3	16.67 ± 6.67
13	1	20.00

Figure 16

Labelled Mitoses Curve of

Early Keratoacanthoma

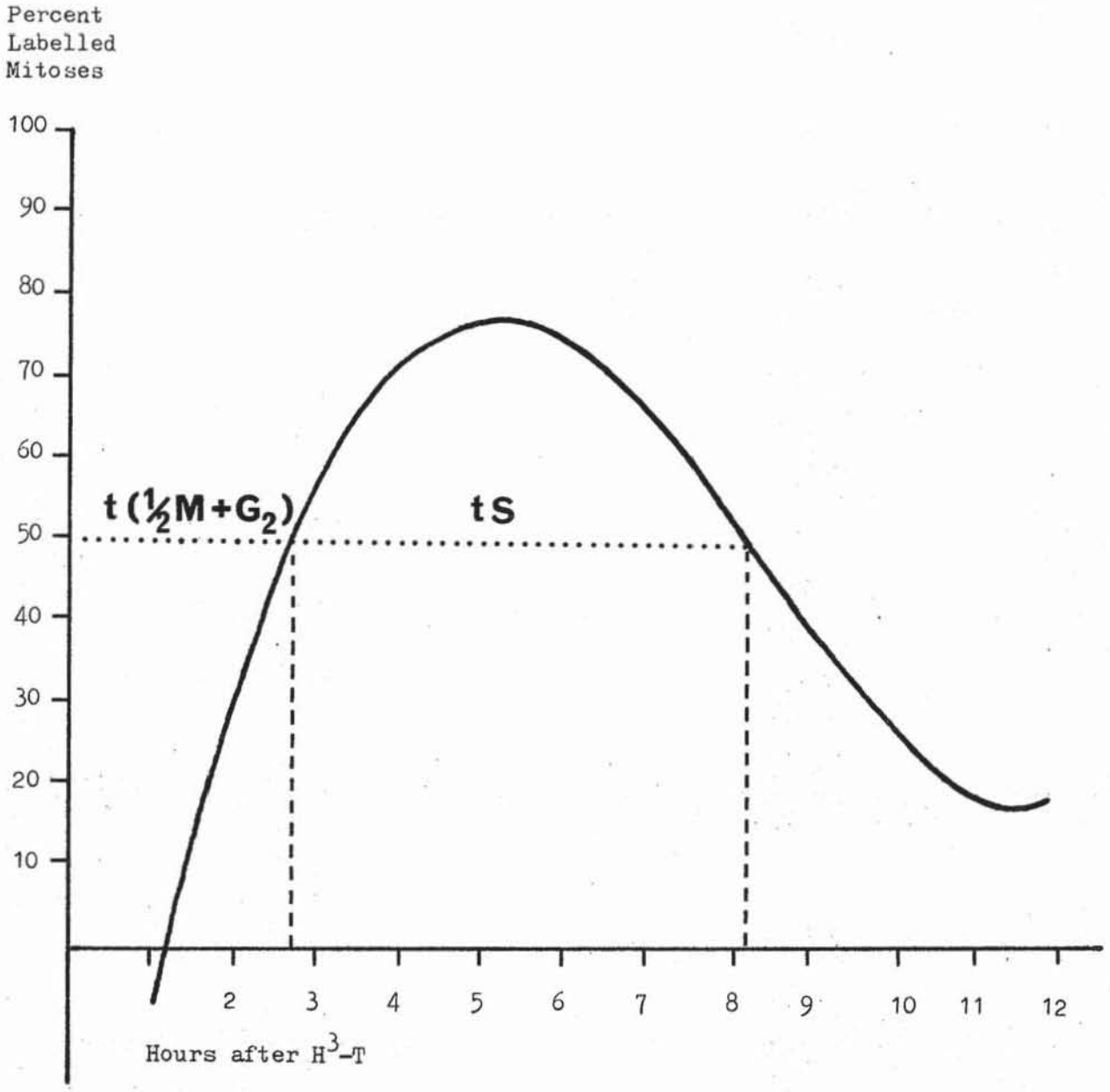


Table 19

Cytokinetics of Early Keratoacanthoma

tC	= 29.38 ± 10.11 hours
tG_1	= 20.89 ± 10.13 hours
tS	= 5.46 ± 0.04 hours
tG_2	= 2.79 ± 0.29 hours
tM	= 0.35 ± 0.07 hours
MI	= 1.65 ± 0.27 %
nS	= 17.74 ± 5.37 %

* Legend see Table 3.

Table 20

Experimental Labelled Mitoses Curve of

Mature Keratoacanthoma

Hours after H^3-T	N	Percent labelled mitoses
1	4	0.00 ± 0.00
2	4	25.75 ± 1.49
2.5	2	17.00 ± 3.00
3	3	53.33 ± 7.51
3.5	4	72.75 ± 9.01
4	3	84.67 ± 8.19
5	4	73.00 ± 9.17
6	3	76.33 ± 10.14
7	4	71.00 ± 6.24
8	2	71.00 ± 11.00
9	2	50.25 ± 12.25
10	1	25.00
11	4	15.00 ± 3.42
12	3	14.67 ± 8.11
13	2	2.00 ± 2.00

Figure 17

Labelled Mitoses Curve of

Mature Keratoacanthoma

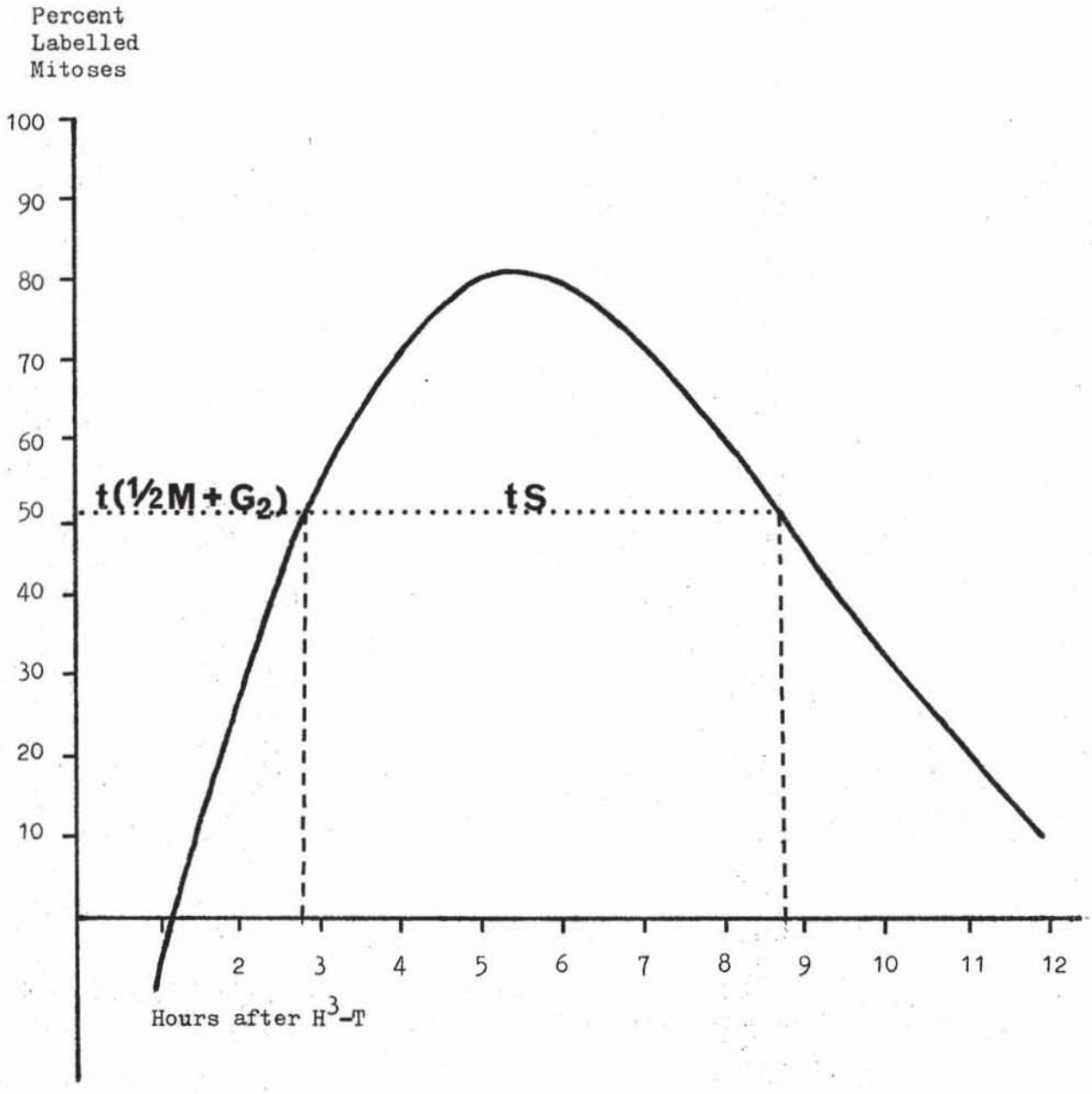


Table 21

Cytokinetics of Mature Keratoacanthoma

tC	= 30.72 ± 7.01 hours
tG_1	= 21.24 ± 6.88 hours
tS	= 5.89 ± 0.15 hours
tG_2	= 2.43 ± 0.22 hours
tM	= 1.26 ± 0.08 hours
MI	= 1.27 ± 0.17 %
nS	= 15.65 ± 3.54 %

* Legend see Table 3.

Table 22

Experimental Labelled Mitoses Curve of

Regressing Keratoacanthoma

Hours after H^3-T	N	Percent labelled mitoses
1	4	0.00 ± 0.00
2	4	18.75 ± 2.84
2.5	4	22.75 ± 4.87
3	4	71.50 ± 10.36
3.5	2	55.50 ± 2.50
4	3	83.33 ± 6.64
4.5	1	60.00
5	4	69.75 ± 2.17
6	4	85.50 ± 5.24
7	1	56.00
8	4	74.75 ± 9.83
9	4	40.00 ± 8.88
9.5	1	40.00
10	3	14.00 ± 4.16
11	2	7.00 ± 3.00
12	2	17.50 ± 9.50
13	1	4.00

Figure 18

Labelled Mitoses Curve of

Regressing Keratoacanthoma

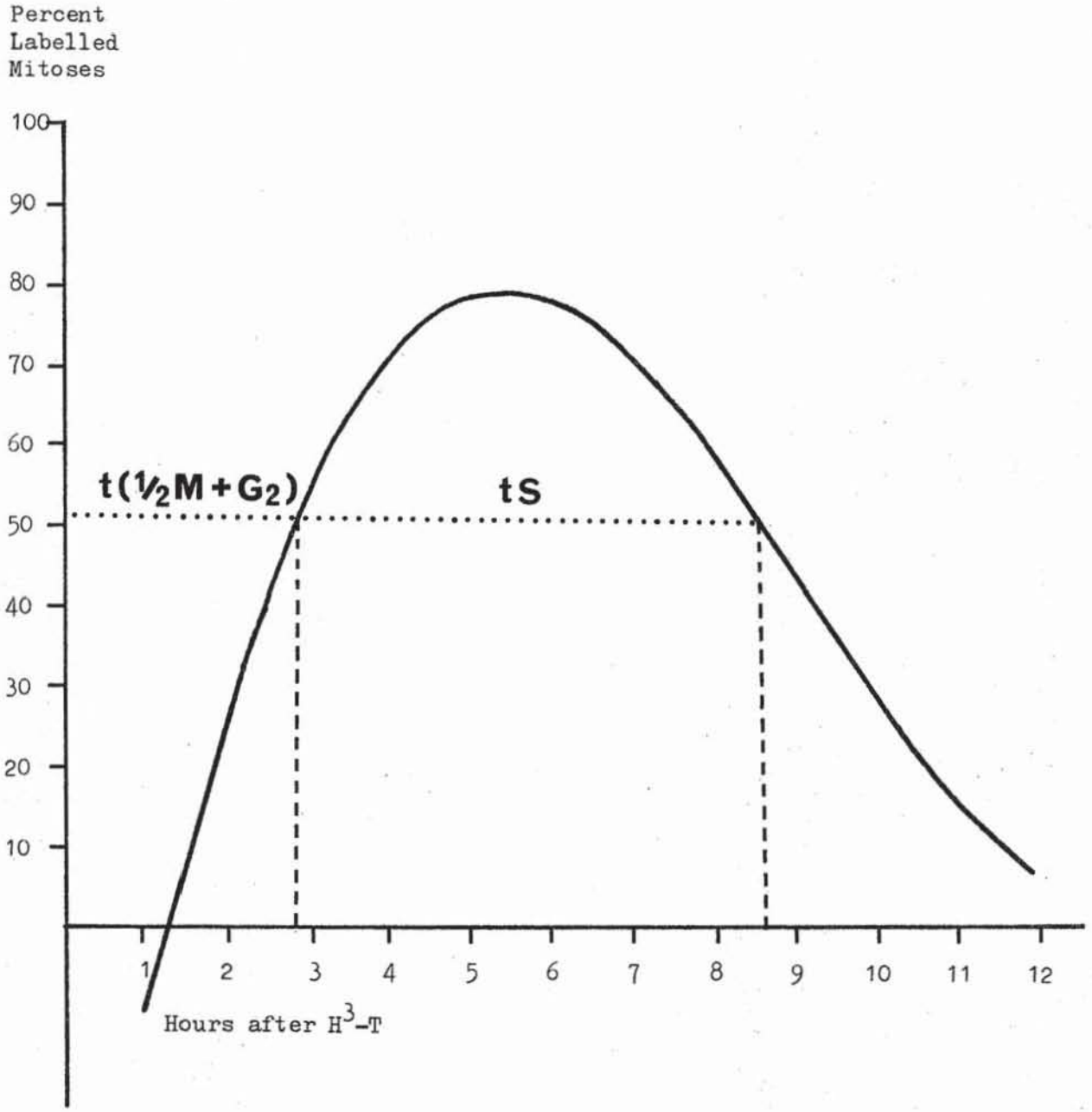


Table 23

Cytokinetics of Regressing Keratoacanthoma

tC	= 27.67 ± 3.59 hours
tG_1	= 17.22 ± 3.60 hours
tS	= 5.69 ± 0.29 hours
tG_2	= 1.49 ± 0.07 hours
tM	= 2.94 ± 0.38 hours
MI	= 2.08 ± 0.36 %
nS	= 14.77 ± 1.52 %

* Legend see Table 3.

Table 24

Experimental Labelled Mitoses Curve of

Squamous Cell Carcinoma

Hours after H^3-T	N	Percent labelled mitoses
1	4	0.00 ± 0.00
2	1	8.00
2.5	4	33.25 ± 5.62
3	3	60.33 ± 13.54
3.5	4	70.50 ± 5.50
4	3	55.00 ± 7.00
5	1	100.00
6	4	70.00 ± 11.75
7	4	80.75 ± 6.97
8	4	69.00 ± 10.38
9	4	42.25 ± 18.67
10	3	12.33 ± 9.06
11	4	8.75 ± 2.98
12	2	14.50 ± 10.50
13	2	6.50 ± 4.50

Figure 19

Labelled Mitoses Curve of

Squamous Cell Carcinoma

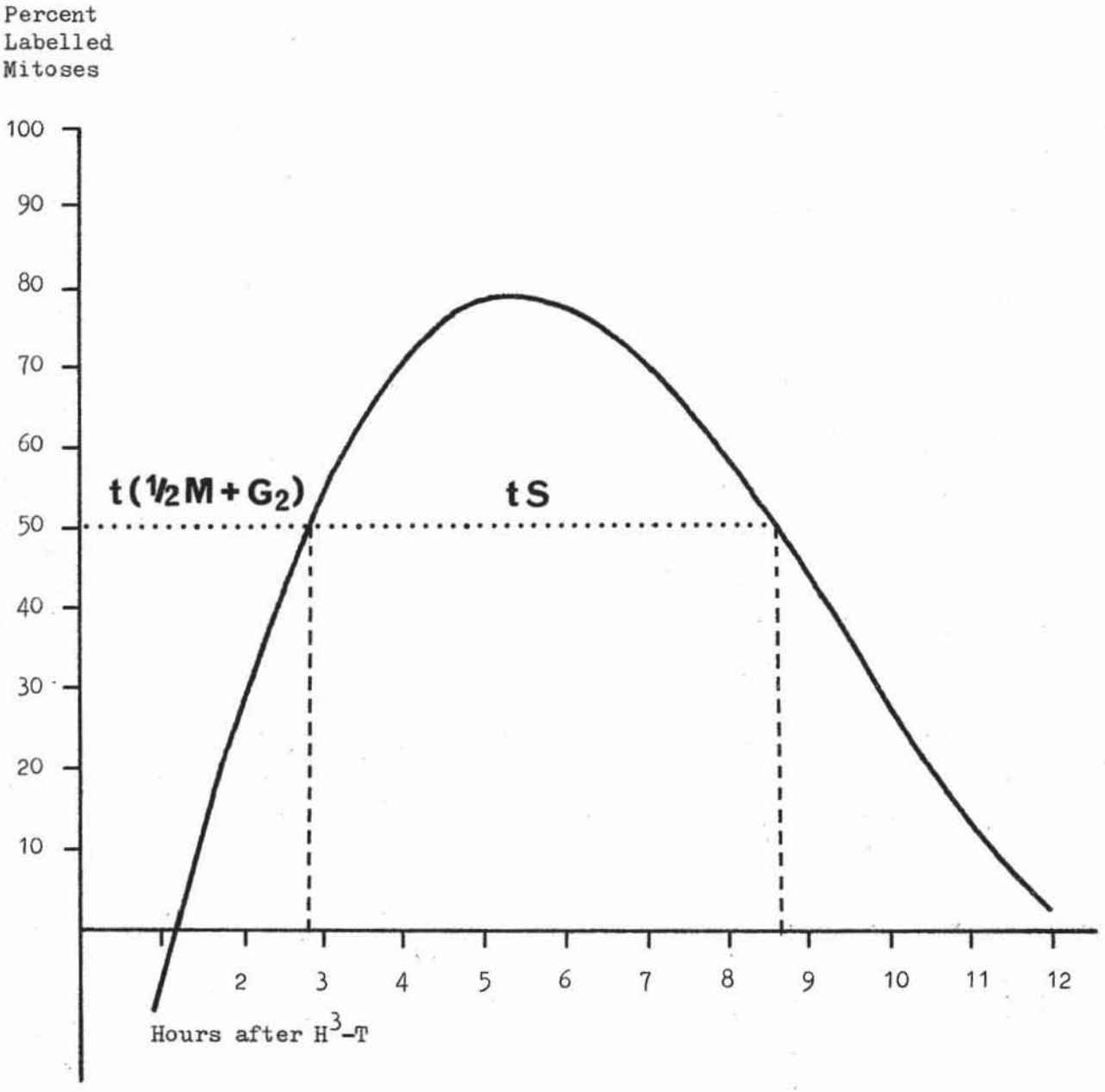


Table 25

Cytokinetics of Squamous Cell Carcinoma

tC	= 41.76 ± 14.63 hours
tG_1	= 32.35 ± 14.82 hours
tS	= 5.70 ± 0.32 hours
tG_2	= 2.38 ± 0.13 hours
tM	= 1.32 ± 0.80 hours
MI	= 0.73 ± 0.06 %
nS	= 13.12 ± 3.66 %

* Legend see Table 3.

Figure 20

Carcinogenic Indices

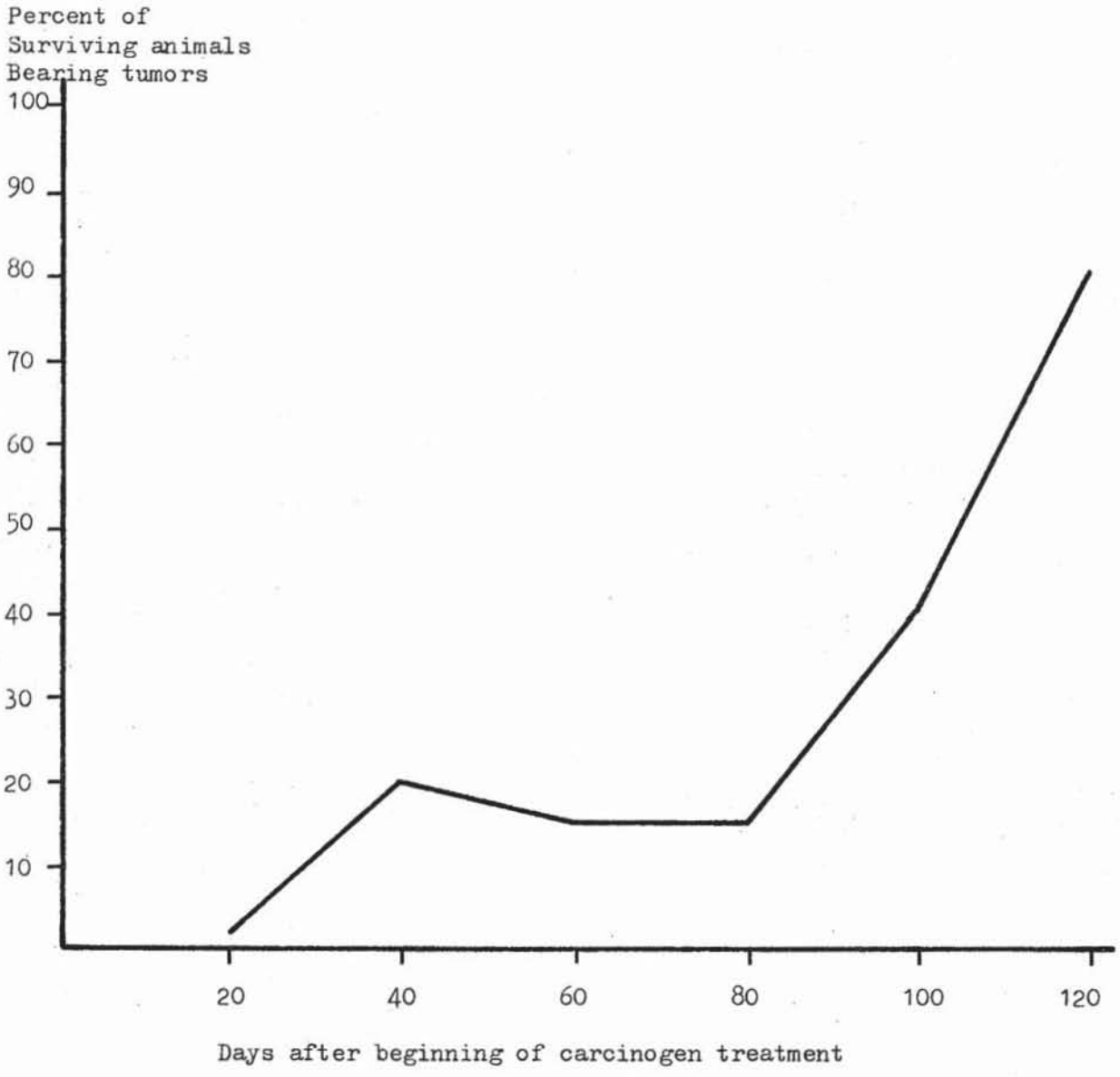


Table 26

Amount of Carcinogen Used

Time	Total amount of Carcinogen Used (per mouse)
20 days*	1.5 mgm BP in 0.30 ml. Benzene
40 days*	2.4 mgm BP in 0.48 ml. Benzene
60 days*	3.3 mgm BP in 0.66 ml. Benzene
80 days*	4.2 mgm BP in 0.84 ml. Benzene
100 days*	5.1 mgm BP in 1.00 ml. Benzene
120 days*	6.0 mgm BP in 1.18 ml. Benzene

* after beginning of carcinogen treatment

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each application contained 150 µgm BP in 0.03 ml Benzene

10 applications in Telogen - 1500 µgm BP

- 1.5 mgm BP in 0.30 ml Benzene

2 applications each week - 0.3 mgm BP in 0.06 ml Benzene

20 day intervals - 0.9 mgm BP in 0.18 ml Benzene

are made here only of those date which occurred between groups.

When all the physiological and pathological conditions taken into account in this study (Tables 27 to 33) were grouped together and subjected to analysis of variance, each parameter (tC, tG_1, tS, tG_2, tM, nS and MI) displayed a variability at a probability level of p= 0.005.

Generation Times

The generation time of normal IFE in Anagen VI was longer than the tC of IFE 40 days after the beginning of carcinogen treatment (p= 5%) [Table 27]. Apart from that difference, the tC of the untreated IFE did not differ from the tC of the treated IFE. The tC of each of the tumors were less than the tC in all other conditions (p= 1%) except that of IFE 40 days after beginning of carcinogen treatment; the latter was not significantly different.

Duration of G_1

tG_1 of IFE in Anagen VI was longer than the tG_1 of IFE 40 days after the initiation of carcinogen treatment (p= 1%) [Table 28]. tG_1 of the induced tumors was shorter than the tG_1 in all other investigated conditions (p= 1%) except IFE at 40 days which was not statistically different. As the statistical analysis indicated the same pattern, the variation of tC was probably brought about primarily by the variation of tG_1.

Duration of DNA Synthesis

IFE in Telogen exhibited the longest tS of all the conditions studied (p= 1%) [Table 29]. tS of IFE in Anagen VI was shorter than the tS of treated IFE on days 20, 40, 120 (p= 1%), as well as of MKA, RKA and SCC (p] 5%). The tS of IFE on days 20 was longer than the tS of EKA (p= 5%), #1 tS of IFE at day 40 was longer than tS of EKA (p= 1%),

RKA and SCC (p= 5%). tS of IFE at day 80 was shorter than the tS of MKA (p= 1%), RKA and SCC (p= 5%). tS of IFE at day 100 was shorter than the tS of EKA (p= 5%), MKA, RKA, and SCC (p= 1%). tS of IFE at day 120 was longer than the tS of the tumors (EKA, RKA and SCC at p = 1% and MKA at p= 5%). It is noticable that the longest tS encountered was 8.35 ± 0.24 hours in normal IFE in Telogen. Also, the shortest tS was not different from normal Anagen VI IFE (4.77 ± 0.22 hours). Thus the greatest range of values of tS occurred in the untreated epidermal cell populations.

Duration of G_2

tG_2 of Telogen was shorter than the tG_2 of IFE at days 60, 80, 120 of treatment (p= 1%), whereas the tG_2 of IFE Anagen VI was longer than the tG_2 of IFE on day 40, EKA (p= 5%), as day 100, MKA, RKA and SCC (p= 1%) [Table 30]. tG_2 of the EKA was shorter than that of the Anagen VI IFE, IFE at days 60 and 80 (p= 5%) and at day 120 (p= 1%). tG_2 of RKA was shorter than the tG_2 at Anagen VI, days 20, 40, 60, 80, 120 (p= 1%), Telogen and day 100 (p= 5%).

Duration of Mitosis

The mitotic durations of all conditions investigated are listed in Table 31. tM of both control groups were longer than the tM of treated IFE on days 20, 80 and 120 (p= 5%), and day 100 (p= 1%). In both of these untreated cell populations the tM was longer than the tM of the EKA (telogen p= 5%, Anagen VI p= 1%). The duration of mitosis of the RKA was longer than in all other conditions, at p= 1%, except in Anagen VI which was not different. The tM of both the MKA and squamous cell carcinoma were longer than that of day 100 (p= 5%). Also, both the tM of day 40 and of day 60 were longer than the tM of the EKA

(p= 5%).

Mitotic Index

The mitotic indices of the control and hyperplastic cell populations were not statistically different (Table 32). The MI of the benign tumors were greater than both the control and hyperplastic MI (p= 1%). The MI of squamous cell carcinoma was greater than the MI of Telogen, days 80, 100 and 120 (p= 5%).

Fraction of Cells in S Phase

The date of the nS are presented in Table 33. The nS of day 40 was not different from the nS of the squamous cell carcinoma but differed from EKA (p= 1%), MKA and RKA (p= 5%). Apart from that exception to day 40, the nS of the tumors was greater than the nS in all other conditions studied (p= 1%). The nS of the control and treated IFE was not statistically different, apart from day 40 when the nS was greater than the nS of Anagen VI.

Table 27

Comparison of Generation Times (in hours)

Telogen IFE	174.65 ± 6.62
Anagen VI IFE	255.25 ± 53.17
20 days*	187.5 ± 29.8
40 days*	119.7 ± 44.0
60 days*	210.7 ± 77.4
80 days*	196.7 ± 24.7
100 days*	234.2 ± 49.8
120 days*	203.4 ± 27.3
early keratoacanthoma	29.38 ± 10.11
mature keratoacanthoma	30.73 ± 7.01
regressing keratoacanthoma	27.68 ± 3.59
squamous cell carcinoma	41.76 ± 14.63

* after beginning of carcinogen treatment

Table 28

Comparison of Durations of G_1 (in hours)

Telogen IFE	161.82 ± 6.81
Anagen VI IFE	245.90 ± 52.87
20 days*	177.6 ± 30.0
40 days*	109.1 ± 44.0
60 days*	203.3 ± 80.0
80 days*	187.4 ± 24.2
100 days*	222.5 ± 45.6
120 days*	197.4 ± 28.9
early keratoacanthoma	20.90 ± 10.13
mature keratoacanthoma	21.24 ± 6.88
regressing keratoacanthoma	17.23 ± 3.60
squamous cell carcinoma	32.35 ± 14.82

* after beginning of carcinogen treatment

Table 29

Comparison of Duration of S (in hours)

Telogen IFE	8.35 ± 0.24
Anagen VI IFE	4.77 ± 0.22
20 days*	6.35 ± 0.25
40 days*	6.72 ± 0.34
60 days*	5.33 ± 0.27
80 days*	4.65 ± 0.31
100 days*	4.47 ± 0.58
120 days*	6.90 ± 0.44
early keratoacanthoma	5.46 ± 0.04
mature keratoacanthoma	5.89 ± 0.15
regressing keratoacanthoma	5.69 ± 0.29
squamous cell carcinoma	5.70 ± 0.32

* after beginning of carcinogen treatment

Table 30

Comparison of Durations of G_2 (in hours)

Telogen IFE	2.39 ± 0.10
Anagen VI IFE	3.73 ± 0.16
20 days*	2.95 ± 0.43
40 days*	2.71 ± 0.33
60 days*	3.87 ± 0.15
80 days*	3.58 ± 0.18
100 days*	2.35 ± 0.40
120 days*	3.92 ± 0.44
early keratoacanthoma	2.79 ± 0.27
mature keratoacanthoma	2.43 ± 0.22
regressing keratoacanthoma	1.49 ± 0.07
squamous cell carcinoma	2.38 ± 0.13

* after beginning of carcinogen treatment

Table 31

Comparison of Mitotic Durations (in hours)

Telogen IFE	1.58 ± 0.31
Anagen VI IFE	1.89 ± 0.43
20 days*	0.59 ± 0.11
40 days*	1.55 ± 0.38
60 days*	1.35 ± 0.33
80 days*	0.59 ± 0.25
100 days*	0.15 ± 0.03
120 days*	0.44 ± 0.11
early keratoacanthoma	0.30 ± 0.07
mature keratoacanthoma	1.26 ± 0.08
regressing keratoacanthoma	2.78 ± 0.38
squamous cell carcinoma	0.98 ± 0.80

* after beginning of carcinogen treatment

Table 32

Comparison of Mitotic Indices (in %)

Telogen IFE	0.19 ± 0.04
Anagen VI IFE	0.35 ± 0.06
20 days*	0.29 ± 0.01
40 days*	0.35 ± 0.06
60 days*	0.51 ± 0.08
80 days*	0.21 ± 0.09
100 days*	0.19 ± 0.03
120 days*	0.19 ± 0.01
early keratoacanthoma	1.65 ± 0.27
mature keratoacanthoma	1.27 ± 0.17
regressing keratoacanthoma	2.08 ± 0.36
squamous cell carcinoma	0.73 ± 0.06

* after beginning of carcinogen treatment

Table 33

Comparison of Labelling Indices (in %)

Telogen IFE	3.06 ± 0.13
Anagen VI IFE	1.20 ± 0.24
20 days*	2.46 ± 0.25
40 days*	7.67 ± 1.78
60 days*	1.76 ± 0.59
80 days*	1.45 ± 0.39
100 days*	1.35 ± 0.32
120 days*	2.59 ± 0.58
early keratoacanthoma	17.74 ± 5.37
mature keratoacanthoma	15.65 ± 3.54
regressing keratoacanthoma	14.77 ± 1.52
squamous cell carcinoma	13.12 ± 3.66

* after beginning of carcinogen treatment

Table 34

Estimated Functions of the Mean Labelled Mitoses Curves

	df =	F =	level of significance
Telogen IFE			
$f(x) = -2.1x^2 + 39.0x - 42.6$	2,42	78.2	0.025
Anagen VI IFE			
$f(x) = -0.06x^3 + 3.93x^2 + 1.19$	2,34	37.3	0.05
20 days*			
$f(x) = 0.023x^4 - 0.467x^3 + 30.18x - 33.9$	3,44	27.9	0.01
40 days*			
$f(x) = -0.013x^3 - 0.32x^2 + 27.7x - 35.6$	3,39	38.7	0.01
60 days*			
$f(x) = 329.6x^3 + 5.708x - 27.02$	2,33	29.75	0.05
80 days*			
$f(x) = -0.426x^3 + 4.625x^2 - 4.39$	3,37	46.8	0.005
100 days*			
$f(x) = 0.0078x^4 + 0.115x^3 + 30.24x - 36.35$	3,40	27.74	0.01
120 days*			
$f(x) = -2.1x^2 + 30.5x - 37.9$	2,41	54.17	0.025
Early Keratoacanthoma			
$f(x) = 0.014x^4 - 6.22x^2 + 56.49x - 58.5$	3,41	27.03	0.01
Mature Keratoacanthoma			
$f(x) = 0.010x^4 - 5.30x^2 + 51.5x - 52.3$	3,44	61.23	0.005
Regressing Keratoacanthoma			
$f(x) = 0.010x^4 - 5.70x^2 + 54.6x - 59.2$	3,44	43.74	0.005
Squamous Cell Carcinoma			
$f(x) = 0.009x^4 - 5.38x^2 + 52.1x - 54.1$	3,43	30.52	0.01

* after the beginning of carcinogen treatment

df = degrees of freedom

F = F ratio from the analysis of variance

CHAPTER V

DISCUSSION OF RESULTS

1) Critique of the Analysis of the Labelled Mitosis Curve

By analysing the labelled mitoses curve it was feasible to estimate the period the average epidermal cell spends replicating its DNA. The procedure employed in this study to describe the labelled mitoses curve presupposes various factors. Firstly, it had to be assumed that in all physiological and pathological conditions investigated, the percentage of cells becoming labelled after a pulse label of tritiated thymidine within a definite period of time described a definable function [f(x)]. Other authors suggested that in a perfect system (ideally a closed system as suggested by L. Bertalanffy, 1960), the function is described not by a curve but by a rapid rise of labelled mitoses, followed by a plateau of a duration equalling tS, ensued by a rapid decline. The entire phenomenon is repeated as cells enter the next cycle. The same author suggested that in most experimental situations the function is a curve with a fairly rapid sigmoid rise and a plateau followed by a more gentle decline.

This is compatible with the view that biological systems are in fact "open" (L. Bertalanffy, 1960), and that this type of curve describes part of a cubic or higher function. The second assumption was that this function [f(x)] can be estimated by the least squares method and that it is one of the following possibilities:

a quadratic equation;

$$f(x) = ax^2 + bx + c$$

$$f(x) = ax^2 + c$$

a cubic equation;

$$f(x) = ax^3 + bx^2 + cx + d$$

$$= ax^3 + cx + d$$

$$= ax^3 + d$$

quartic equation;

$$f(x) = ax^4 + bx^3 + cx^2 + e$$
$$= ax^4 + bx^3 + dx + e$$
$$= ax^4 + dx + e$$
$$= ax^4 + e$$

or higher function;

$$f(x) = ax^5 + bx^4 + cx^3 + d$$

In order to facilitate a close estimate of the regression equation, as many values were determined per time interval as it was practically feasible (usually 4).

The functions of each of the above possibilities were thus appraised, and the one with the lowest standard error of estimate was selected as the function that fitted the curve most accurately. Once the power of the function was known, each set of four values per time period were employed to calculate the four equations for each experiment.

Each set of data (1/4 of the total number) was fitted to the power of the curve of the entire set, and the estimated values of this second curve were plotted.

For instance, for epidermis in Telogen, the best fitting equation of all the data available was $f(x) = 2.7x^2 + 39.0x - 42.6$ with $F = 78.21$ and $df = 2.42$.

The four separate equations of 1/4 of the data for Telogen were:

mouse 1 $f(x) = -2.8x^2 - 39.9x - 40.5$

mouse 2 $f(x) = -2.3x^3 + 34.3x - 32.2$

mouse 3 $f(x) = -3.3x^2 + 46.4x - 63.1$

mouse 4 $f(x) = -2.3x^2 + 34.4x - 30.6$

The values of $t(1/2\ M + G_2)$ and tS were estimated from these final four equations. As four values were available, an estimate of the variance (σ) was feasible and thus comparisons could be made on a statistical basis.

The values of tS and $t(1/2\ M + G_2)$ are interesting, yet they are not particularly useful unless they can be associated with a meaningful expression of variance (σ) and that the method by which the variance is ascertained is in fact valid. It is most important that the derived functions [f(x)] (Table 34) represent a true correlation of the percentage of labelled mitoses versus time. It is essential to know whether or not the functions thus derived accounted for most of the variability of the percentage labelled mitoses versus time. Employing the null hypothesis that: the data did not describe a curve and an alternate hypothesis that; the date did describe a curve, we might determine whether a curve fit the data if the experimental F ratios were greater than the theoretical F ratio.

The theoretical levels of F at the following degrees of freedom and levels of significance were:

for df = 2, 30;

p = 0.05	F = 19.46
p = 0.025	F = 39.46
p = 0.01	F = 99.46
p = 0.005	F = 199

and for df = 2, 40;

p = 0.05	F = 19.47
p = 0.025	F = 39.47
p = 0.01	F = 99.48
p = 0.005	F = 199

and for df = 3, 40;

p = 0.05	F = 8.59
p = 0.025	F = 14.04
p = 0.01	F = 26.41
p = 0.005	F = 42.31

taken from Table A 14 of Snedecor and Cochran, 1967.

The F ratios derived from each of the twelve experimental curves were larger than the theoretical F ratios, at least at p = 0.05.

Therefore the null hypothesis was rejected and the alternative hypothesis accepted, that is, that there was a curve described by each set of data.

In order to estimate the durations of $t(M/2 + G_2)$ and tS accurately, these values should be calculated from a function in which time is the dependent variable, whereas the percentage of labelled mitoses constitutes the independent variable. In other words, a function where the percentage of labelled mitoses is known without error, and time varies as a function of that percentage of labelled cells. Unfortunately, in the present set of experiments, time constituted a very minor error (merely 5 - 10 minutes at the most), and thus had to be considered the independent variable, whereas the percentage labelled mitoses constituted the dependent variable, in accordance with variations inherent to biological phenomena.

Therefore, there remained the choice of introducing either one of two errors. Either the percentage labelled mitoses was considered the independent variable and the measurements of time (and also tS and $t[M/2 + G_2]$) were correct; alternatively, time was the independent variable and the percentage of labelled mitosis was measured correctly to yield an invalid estimate of tS and $t(M/2 + G_2)$.

Of these two alternatives, it appeared more reasonable to select the second course and to assume that the estimations of tS and $t(M/2 + G_2)$ were accurate. The reason for this choice was because it suited more appropriately the biological conditions.

In conclusion, the suppositions that had to be made in the analysis of the labelled mitosis curves were: 1) the percentage labelled mitoses versus time described a function $[f(x)]$; 2) the function $[f(x)]$ could

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be determined by the mean square error method; 3) this function was one of twelve functions tested above; 4) the entire data can be assumed to describe accurately the function of the labelled mitosis curve under a certain set of circumstances; 5) each set of data (1/4 of the total) could be used independently to estimate that function (with the same characteristic of the curve of all the data); 6) time was an independent variable; 7) the estimates of tS and $t(M/2 + G_2)$ derived thusly were, in fact, accurate; and 8) that at each time interval the percentage labelled mitoses was distributed normally.

In a study of this nature one must not lose sight of the assumptions upon which the labelled mitosis curve is based. Specifically, that the population is asynchronous and that the cells that are in the cycle pass from one phase to the next independently. The assumption that cells are distributed equally throughout the cell cycle is not valid for cell populations that grow exponentially, although it is probably true for cell populations where the growth pattern is linear. Linearly growing cells that enter the cycle repeatedly must thus be kept apart from those cell populations where cells will not reenter mitosis. Only the intestinal epithelium has been observed to exhibit these latter characteristics (Siskin, 1964).

The problem of asynchrony and carcinogenesis is a major one. By observing a possible cycling effect in the mitotic rates of IFE treated with BP while in Telogen during the first thirty days (Fig. 13, P. 60 of Chivers, 1967), one comes to wonder whether partial synchrony constitutes a major or minor factor in carcinogenesis, or whether it plays any significant role at all. Cyclic phenomena were also evident from the mitotic indices of (hr/hr) hairless male mouse IFE, treated once

with BP (Fisher and Chivers, 1969, unpublished data). When comparing the specific activity of DNA (counts per minute tritiated thymidine per microgram of DNA) after different molar concentrations of the A_1 fraction of Croton Oil (Raick, 1969, unpublished data) some medium level concentrations produced cyclic-like phenomenon. High doses caused inhibition of DNA synthesis lasting for a long period, while low doses produced an initial decline in DNA replication, lasting merely a few hours, followed by a rebound activity which slowly returned to normal levels. It appeared from these observations that cyclic activity in cocarcinogenesis was dose dependent, but did not play a major role in carcinogenesis. Although dose concentrations were comparable, such a dose relationship is not known to exist with BP or DMBA.

Diurnal variations of mitotic rates can also be considered as expressions of partial synchrony. However, as all experiments in this study were carried out during strictly controlled and identical time periods, this phenomenon should not have affected the data. Moreover, possible seasonal differences exist. Such have not been adequately studied in the mouse, and it was assumed that they were inconsequential to this study. All mice were housed under constant temperature, and humidity conditions with a rigidly controlled lighting schedule.

In an ideal system the labelled mitosis curve should attain 100%. However, in the actual estimation of the experimental values the curve does not usually reach, or need to attain the 100% level. Failure to attain that level may be ascribable to a lagging of some cells with replicated DNA before entering mitosis, to some cells remaining halted in G_2 for an exceptionally long period (as G_2 derived G_o cells), or to occasional unlabelled cells passing from G_1 to mitosis usually quickly

(Siskin, 1964). In the present study, the percentage of labelled mitoses did not rise very high (Fig. 8) in the Anagen VI IFE. The duration of mitosis (tM) was quite long, and it is conceivable that cells were entering G_2 where they remained longer in Anagen VI than cells in the Telogen stage.

Throughout this thesis, the data reporting the various observations were presented to the second decimal place. This procedure was employed to standardize the utilization of the computer. There should be no delusions that the values thus reported were considered to be that accurate, however. For example, tC of Telogen was reported to last 175.65 ± 6.62 hours; this figure is actually to imply that the tC lasted about 175 ± 7 hours. And even that rounded off data was probably not too precise. Also, the generation times and tG_1 could be interpreted for practical purposes, to range from 8 to 10 days. The MI and nS data are probably as accurate as reported, whereas all other figures indicated were presumably one, or even two, significant numbers too accurate.

Elsewhere in this report, evidence is presented suggesting that the four-stage cell cycle is probably incorrect. Indications exist that there occurred at least two non-proliferating cell compartments in the normal epidermis, conceivably G_o cells derived from both the G_1 and G_2 phases. Pertinent to this is Mendelsohn's (1965) statement that "once one has accepted the concept of non-proliferating tumor cells, one is committed to the proposition that cell cycle time cannot be estimated from growth curves, mitotic index determinations, or techniques such as colchicine blockade and accumulation of mitotic figures as a function of time". However, the two methods employed in this investigation may still be valid to serve as a mean population parameter, as the calculation

method is based on nS, tS, and the growth fraction (assumed to be N = ln2), and the ratio method is based on cell differentiation rather than proliferation.

2) Critique of the Statistical Data

Having proceeded this far in the report and accepted the labelled mitoses curve and its analysis, an attempt must be made to sift the actual biologically significant data from the merely statistically data and premises. The following section is thus an attempt to segregate the biologically (or pathologically) important facts , which are further discussed in the subsequent section.

Generation Time

The procedure utilized for the estimation of the generation time was not entirely satisfactory. It was based on two other parameter (tS and MS) that contributed to the variation of the estimated tC. There seemed to exist basically two durations of the generation time, namely that of the normal epidermis which (with one exception, day 40 after hair plucking) included the treated and thus hyperplastic IFE as well, and secondly, the tC of the tumors. The normal and hyperplastic IFE exhibited a 6 - 7 fold longer tC than the tumor cells. The generation times of the tumors were shorter simply because more cells were proliferating at any one time.

Duration of G_1

As mentioned previously, the statistical variability of tG_1 displayed a pattern incidental to the tC. Thus most of the variability of tC was in turn affected by the variability of tG_1.

Duration of S

As the range of the times required by cells to synthesize DNA was

greatest within the untreated epidermis (for instance, Anagen VI tS = 8.35 ± 0.24 hours, or Telogen tS = 4.77 ± 0.22 hours) and the tS on days 80 and 100 of carcinogen treatment were not different from that of Telogen, one may conclude that epidermal tS did not vary significantly in the course of benzopyrene induced carcinogenesis in the mouse.

Duration of G_2

If the tG_2 of the regressing keratoacanthoma is excluded, it became evident that tG_2 of normal epidermis in Telogen (2.4 hours) and Anagen VI (3.7 hours) yielded the extreme low and high values. The development of benign or malignant tumors seemingly did not affect the duration of G_2. tG_2 became shorter only as the benign tumor was regressing. Inasmuch as the RKA exemplified a special condition of a tumor in self-destruction, the tG_2 of its cells cannot readily be compared with that phase of the cycle variably proliferating cells, whether normal or malignant.

Duration of Mitosis

Except for the RKA, the longest tM was noted in the normal (control) cell populations. All other values of tM were shorter than these control values either statistically (as on days 20, 80, 100, and 120, EKA, MKA and SCC), or absolutely (days 40 and 60). The RKA was again considered as a special case, as this tumor was in the process of destroying itself by means unknown. Thus, its cells passed through mitosis apparently with difficulty only.

Mitotic Index

The MI of the BP treated IFE remained within the range of normal values. It was anticipated that the carcinogen treated cell population would exhibit a decreased mitotic duration (based on the observations of

Bullough and Laurence) as the cells were in mitosis for a shorter time and more cells should enter mitoses. Yet, this expectation was not realized in the benzopyrene treated mouse IFE as the tM decreased but the MI remained at normal levels.

The MI of the benign tumors was larger than that of either the hyperplastic and untreated IFE by a minimum factor of 3 to 6. The MI of the RKA was higher in accordance to the increased tM of that tumor. The MI of the SCC lasted longer than MI of Telogen (statistically) and of Anagen VI (apparently).

The highest MI was observed in the RKA. This could be interpreted as indicating that these cells completed mitosis only with considerable difficulty as they also remained longest in that phase as compared to the other cell populations.

The tM of the EKA was considerably shorter while its MI was larger in comformity with the chalone theory. The MI of MKA was greater while its tM remained at almost normal levels. In the special case of RKA, there occurred a long tM and more cells were in mitosis at any time. The SCC displayed statistically normal tM and MI.

In the benign KA'S a considerable increase occurred of the MI in all stages of development; yet only the EKA exhibited a shorter tM, whereas the tM of MKA remained almost normal, and that of the RKA was grossly prolonged.

Fraction of Cells in S Phase

Apart from on day 40, the nS of the treated IFE remaining within the normal control range at all times. The nS of the tumors was 4 - 6 times higher than the nS in other stages (except day 40).

Summary

1) tC decreased 6 - 7 fold at tumor development.

2) tG_1 decreased 6 - 7 fold at tumor development.

3) tS, tG_2 remained unchanged throughout carcinogenesis.

4) MI increased at tumor development, particularly in the benign tumors.

5) nS increased at tumor development.

6) Between the beginning of carcinogen treatment until the development of tumors, the duration of mitosis became much reduced.

7) 40 days after the beginning of the carcinogen treatment the tC, tG_1 and nS were different both from the untreated IFE values as also from those of the tumors.

8) The durations of mitosis during the second half of carcinogen treatment became reduced by 2 - 8 fold.

3. Discussion of IFE at 40 Days of Carcinogen Treatment

IFE, treated with Benzopyrene but not yet bearing tumors, exhibited a pattern of cytokinetics remarkably similar to that of the normal IFE. There was only one exception, the IFE on day 40 of BP treatment. In the latter instance the tS, tG_2, tM, and MI remained close to normal but the nS was twice that of normal IFE, whereas the tC as well as the tG_1 were considerably shortened. One is tempted to argue that the date of the tC and tG_1 were based primarily on the estimate of nS (as tS was relatively constant) and as such was artificial; however this experiment, by chance, was the only one which utilized two different methods of estimating tC. If the estimate of tC from the calculation of nS and tS was unrealistic, it would be highly improbably that an alternate estimate of tC would agree to it. tC of IFE at 40 days according to calculation was 119 hours, whereas

estimation of the rate of exit of cells from the basal layers (transit time) yielded 128 hours; both data were therefore fairly close. Not only was the situation of the hyperplastic IFE at day 40 different from the cytokinetics of other IFE, whether normal or hyperplastic, but the extimate was thus also reliable. It is possible to correlate that point (day 40) with the approximate half-way mark between the initiation of BP application until the appearance of the majority of the tumors.

The actual reason as to why the day 40 IFE did not conform with the cytokinetic pattern of the untreated or hyperplastic IFE is unknown.

4. Discussion of the Cytokinetics of Regressing Keratoacanthoma

Most of the parameters (tC, tG_1, tS, and nS) studied in the regressing keratoacanthoma (RKA) were similar to those ascertained for the other tumor types. Yet, of all varieties of IFE, whether normal, hyperplastic or neoplastic, the RKA exhibited the highest mitotic index (MI), the longest mitotic duration (tM), and the shortest tG_2 phase (duration of the premitotic gap). Is it conceivable that this cell population passed through G_2 exceedingly quickly, thereby failing to produce sufficient quantities of a certain chemical or of several materials (RNA, protein, ATP) requisite for the normal mitotic processes? If this were true, this circumstance could well have retarded the subsequent passage of cells through mitosis, more mitotic cells would be present at any instant, (and responsible for the increased MI) in the RKA, which was higher than that of cell populations with a considerably shorter mitotic phase. The RKA differed from the other tumor types by the circumstance that its cells were engaged to a greater extent in differentiation than those of other tumors. If this process of differentiation would have proceeded at a rate only slightly faster, this tumor

might have regressed completely, eventually leaving in its wake merely a small scar. This regression of the tumor to a mere scar was the course that the RKA almost achieved. Inasmuch as the RKA were engaged in a self-destructive course, it cannot truly be considered to exemplify a carcinogenic phenomenon.

While the RKA was reverting to a semblence of a normal cell population, it proved very interesting. Why did this tumor approach the normal state whereas the squamous cell carcinoma (SCC) presented a progressively poorer prognosis? Part of the reason apparently was the circumstance that the RKA was composed largely of normal cells still limited from the underlying tissue by a preserved basement membrane. In contrast, SCC was characterized by both a lack of the basal lamina, as well as by increased cellular anaplasia. It must be pointed out, however, that a few of these RKA tumors contained areas resembling morphologically, carcinoma-in-situ (Bowen's Disease). This was evidence that many cells or cell groups occurred in instances even within that tumor population that not only did behave differently but were capable of transforming into carcinoma. Without doubt, some of these areas became carcinomas.

5. Comparison Between Present Observations and Previous Reports in the Literature

In a comparative study of the cytokinetics of the normal hamster cheek pouch epithelium and carcinoma induced in that area by DMBA, Reiskin and Mendelsohn (1962, 1964) noted less variation in the duration S phase (tS) between tumor cells than between the cells of the normal pouch epithelium. Moreover malignant tumor growth was associated with a

shortened generation time, particularly a reduction of tG_1. Dormer et al. (1964) reported the generation times of normal IFE of mice (tC = 150 hours), hyperplastic IFE after application of MCA (tC = 56 hours), and the epidermal carcinoma induced by the treatment (tC = 33 hours). From these data, Bresciani (1968) concluded that malignant tumors displayed a shorter tC than their tissue of origin. Dormer et al. (1964) asserted that the mitotic rate and nS became augmented with progressing stages of hyperplasia, at the expense of the tC which declined correspondingly. These authors suggested further that the differentiation phase of the proliferating cell population was being shortened, while the duration of DNA synthesis remained unaltered in the epidermis even after treatment with MCA (0.25% weekly).

Reiskin and Mendelsohn (1962, 1964) applied DMBA to hamster cheek pouch epithelium, while Dormer et al. (1964) treated mouse skin with MCA. Yet both groups of authors arrived at similar conclusions. The latter agree also with the observations in the present study, applying BP to the epidermis of SWR mice. It thus appears that the most significant changes in carcinogenesis are neither dependent on the animal species nor on the chemical composition of the carcinogen.

Mendelsohn (1965) proposed further that "tumors of common origin have common cell cycles, and the results from the hamster study (Reiskin and Mendelsohn, 1964) also indicate that the cell cycle of the tumor may be very different from the cell cycle in the tissue of origin." The experimental model in the present thesis, the epidermal cell population concurred with these assertions to a remarkable extent. Mendelsohn (1965) continued, "perhaps the crucial difference between normal and malignant tissues in that normal cells are subject to modulation in their cell cycles, whereas tumor cells proliferate at a

maximum rate determined by the degree of differentiation, the tissue of origin, or some other feature of the tumor type. Local and systemic restraints continue to operate on the tumor as a whole, determining the growth mode and the fraction of cells proliferating, but to the individual it is an all or nothing situation."

These statements serve remarkably well to explain the observations in the present study. The latter signified that the cytokinetics of the normal IFE was indeed exceptionally variable, whereas that of both the carcinogen treated IFE and benign or malignant tumor types exhibited decreasing variability. It was further observed that the epidermal tumor cells were by no means proliferating at the maximum rates of which other cells are capable, such as those of the intestinal crypts or of the hair matrix, proliferating at exceedingly rapid rates for cell renewal or hair growth, respectively. Yet it is not actually possible to compare between epidermis and intestine, as the latter constitutes an entirely different cell population and its rate of proliferation is governed by completely different factors (such as continual exposure of the cells to proleolytic enzymes). But one is tempted to compare between the hair matrix and IFE, as both are epidermal cell populations; yet they remain still dissimilar as is also their response to external agents. For instance, the hair matrix did not participate in the histogenesis of BP induced epidermal tumors despite the circumstance that its cells were similarly exposed to the carcinogen (Chivers, 1967).

The present report demonstrated further that BP treated cell populations exhibited a seemingly shortened (although not statistically significantly shorter) tC, and = definitely a normal to reduced tM. In fact, both the control and non-tumor bearing treated IFE behaved very

similarly. This is quite reasonable if only a few cells become transformed but most of the treated cell population had been missed by the transformation into latent tumor potentialities. Along these lines Frankfort (1967) postulated that the cancer cells were frequently characterized by accelerated cell proliferation. Tumors which arose from non-renewing tissues, such as liver or neuroglia exhibited an acceleration of cell proliferation. In contrast, tumors that developed from renewing cell populations, as from the IFE, displayed either accelerated or unaltered rates of proliferation.

Both the generation time and tS of IFE proved to be greatly variable in the present study. But also the histology and physiology of different epidermal areas varied considerably from one body area to another. Some of the more conspicuous variations reported in different studies of the IFE can presumably be ascribed to the circumstance that the epidermal specimens were derived from different body regions; particularly the ear IFE is markedly different from the back skin IFE (Wallace, 1964). For instance, the IFE of the mouse ear displayed a tS = 18 hours, tC = 30 to 100 hours (Pilgrim et al., 1966). The tS of guinea pig hyperplastic epidermis remained relatively normal. In the same animals, the tC was longer in hyperplastic IFE (Song and Tabchnick, 1969). After a pulse label, the labelled cells in that study were observed exclusively in the basal layer of the back skin IFE of normal animals, whereas in the hyperplastic IFE, labelled cells occured in the lowermost layers. In human epidermal tumors (after incubation in a solution of H^3-T for 3 - 5 hours) Rashad, (1967) noted the H^3-T label in the basal, spiny and even in the granular cell layers, whereas in the normal IFE, the label occured exclusively in the basal and parabasal cells.

The cell population of carcinoma in situ of the human uterine cervix yielded an essentially homogeneous reaction following a pulse label of H^3-T. The fraction of cells becoming labelled became augmented with increasing anaplasia (Richart, 1963). That author ascertained a generation time of 5.7 days for the normal epithelium of the human uterine cervix which became reduced to a mere 11.3 hours in the cervical carcinoma *in situ*.

The growth kinetics of the human epidermal squamous cell carcinoam were reported as being highly variable: tC = 1 to 4 days and tS = 7 to 19 hours (Frindel et al., 1968); tC = 6 to 9 days, tM = 17 to 92 minutes and tS = 4.6 to 39 hours (Meyer, 1969).

While studying DAB induced neoplastic transformation of rat hepatocytes, Simard and Daoust (1966) observed that it was initially associated with an increased nS. The uptake of tritiated thymidine was enhanced also in extrachromosomal fragments and in pyknotic granules. In the present study an increased nS was associated only with the actual appearance of tumors. It is conceivable, however, that prolongation of S, if it occurred at all, was relatively so slight that it remained undetectable in the samples investigated during the present study.

Titus and Shorter (1965) reported the MI and the nS of *in vitro* labelled human pulmonary squamous cell carcinoma to be 1% and 17%, respectively. These responses were quite similar to those observed also in the SCC of mouse skin in the present investigation.

In an *in vitro* study of human invasive SCC of cervix, Bennington (1969) determined the cytokinetics of two such solid tumors. The mean parameters were: tC = 15 hours, tG_1 = 1.3 hours, tS = 10.6 hours and tG_2 = 1.9 hours. The human uterine SCC's were quite different to the mouse

skin SCC, both physiologically and histopathologically; for one, the human SCC's were of a highly invasive type whereas the mouse skin SCC did not display advanced signs of invasive tendency.

Rashad and Evans (1968) studied the cytokinetics of DMBA induced papilloma and Shope virus papilloma, and concluded that the nS of both tumors was 4 to 6 times greater than the normal dorsal IFE of rabbits. This observation is very similar also to that in the present study.

6. The Effect of Benzopyrene on the Cytokinetics of Mouse IFE

In the course of tumor development, the generation time of the epidermal cell population became reduced 6 to 7 fold, ascribable primarily to a reduction of the duration of G_1. In contrast, the lengths of the S and of G_2 phases were not altered significantly. The mitotic index increased at the time distinct tumors developed, particularly benign tumors. Concurrently, the fraction of cells synthesizing DNA became increased several fold over normal. The basic change during tumor development was that more cells were in the proliferative compartment at any time, at the expense of a much shorter G_1.

It seemed as though the tumorigenic mechanism abandoned some of the more sophisticated growth controlling mechanisms which had been developed during normal differentiation and evolution. Tumor growth might be considered to constitute a more primative mechanism of cell multiplication that either lost its inhibition, or else gained some accelerating stimulus affecting the factor(s) controlling the passage of cells from compartment to compartment of the cell cycle. Conceivably, a tumor may have thus lost part of the inhibitory mechanism that normally regulates the passage of cells from G_1 to S, for instance. Other possibilities are that, under the action of a carcinogen, the

mechanism became altered governing the flow of cells from G_o to S or the return of G_o cells into G_1 phase.

There appear to exist certain contradictory phenomena in the cytokinetics of tumors. For instance, the proportion of nS in the tumors was relatively constant (about 15%), yet the mitotic index was considerably lowered in the SCC (0.73%). An explanation of this observation might be that some malignant cells did not proceed immediately to mitosis after having completed DNA synthesis.

CHAPTER VI

SUMMARY

The present study was concerned with the investigation of the cytokinetics of normal epidermal cell populations, as well as of the same cell communities during carcinogenesis. The principal observations were:

1) An improved method of estimating $t(M/2 + G_2)$ and tS was utilized to enable more accurate comparisons between a variety of variables studied.
2) The range of cytokinetic activity of mouse interfollicular epidermis (IFE) was determined relative to the phases of the hair growth cycle.
3) The durations of S and G_2, as the fraction of cells synthesizing DNA, were significantly different in Telogen and Anagen VI of normal, untreated IFE. In contrast, other parameters (tC, tG_1, tM, nS and MI) were not significantly altered during the hair cycle phases.
4) The initial reaction to carcinogen treatment (20 days after the beginning of benzopyrene application) was a shortening in the mitotic duration.
5) 40 days after carcinogen treatment had been initiated, the fraction of cells synthesizing DNA (nS) became augmented to a value lying between nS of untreated IFE and the nS of the tumor cells. The generation time and tG_1 declined to an extent that they were not different from those of the tumor cells but were dissimilar to those of Anagen VI in normal IFE.
6) The duration of mitosis during the second half of carcinogen treatment (days 80, 100 and 120) decreased by 2 - 8 fold.
7) At the time of tumor development, tC decreased by 6 - 7 fold, chiefly by a shortening of the G_1 phase.
8) The duration of DNA synthesis of the tumor cells was not different

from that of the tS of the normal or non-tumorous benzopyrene treated IFE. However, the individual variability of the tS between the tumors was considerably reduced.

9) The duration of G_2 of the tumors (apart from that of the tG_2 of the regressing Keratoacanthoma) was within the range of that of the untreated IFE cells. tG_2 of the regressing keratoacanthoma was the shortest duration of G_2 encountered.

10) The duration of mitosis of BP treated IFE was quite variable, such variations being equal to those in the normal cell population; but these values of the former cell population was below the normal range.

11) The tM proved extremely variable both between the tumors as within the same tumor state. The regressing Keratoacanthoma displayed the longest tM. The tM of the squamous cell carcinomas was the most variable of any type of epidermal cell population studied.

12) The mitotic indices of the benign tumors were higher than those of the normal control or hyperplastic IFE.

13) The mitotic index of the squamous cell carcinoma was significantly higher than that of normal Telogen epidermis or of the non-malignant treated IFE. However, the mitotic index of Anagen VI in normal epidermis was similar to that of the squamous cell carcinomas.

14) The fraction of cells synthesizing DNA in the tumors was larger than of the cells in all other conditions (except at 40 days after the beginning of carcinogen treatment).

15) A variability was not apparent between the fraction of cells in the S phase of the tumors. The pooled nS of all tumors investigated was $15.32 \pm 1.74\%$.

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APPENDIX

Table A1

Raw Values of Percent Labelled Mitoses at Different Times After Administration of H^3-T to Experimental Animals

	Hours after H^3-T											
Experiment	1	2	3	4	5	6	7	8	9	10	11	12
Telogen	0	17		86	90	92	100	100		50	80	23
IFE	0	0	95	71	71	75	90	84	100	86	75	33
	0	0	25	86	100	85	83	100	100	86	63	0
	0			63	100		100	100	63	100	75	50
Anagen VI	0	29		33	66	43	66	64	50	50	0	
IFE	0	0		42	66	83		50	50	0	0	
	0	0		33	100	71	33	100	75		0	
	0	39	29	20	60	66	63					
IFE at	0	43	75	86	75	71	86	100	43		30	0
20 days*	0	0	20	70	80	50	50	86	100	66	50	0
	0	0	66	85	71	82	100	50	50	11	29	31
	0	20	33	62		78	71	81	25	11	20	
IFE at	0	14	38	40	50	53	92	89	38	64	44	14
40 days*	0	0	27	75	76	87	88	100	30	20	44	0
	0	13	40	48	61		100		70	20		12
	0	11		79			100		71	50		11
IFE at	0	0	9	21	69		58	42		44	13	0
60 days*	0	0	32	63	73	91	98	29		33	0	0
	0	0	30	80	33	69				13		
	0	0	52		30	83				42		
IFE at	0	0	13	36	62	77	66	86	33	10	5	
80 days*	0	33	50	40	78	66	83	100	46	21		
	0	0	0	25	60	71	92		57	43		
	0	10	33	54	50		60		55	40		
IFE at	0	40	33	58	84	83	47	100	40	13	13	0
100 days*	0	15	21	61	67	66	66	54	13	42	0	0
	0	0	33	19	80	60	44	40	0			
	0	21	38		90	80	76	22	39			
IFE at	0	0	0	11	66	50	100	75	66	78	40	33
120 days*	0	16	46	80	100	80	89	71	55	57	40	25
	0	12	14		75	66	74	66	76	29		14
	0	9	28		50		75	66	75			50

Table A1 (cont'd)

	Hours after H^3-T															
riment	1	2	2.5	3	3.5	4	5	6	7	8	8.5	9	9.5	10	11	12	13
y	0	0		90		80	88	82	52	25		52			40	30	
	0		0			92	93	78	86	60		20			15	10	
	0		20			73	81	37	50	90		60			9	10	
	0		40		60	88	88	68	45	69		12			19		20
re	0	25	14		72	82	59	58	70			63			16	28	0
	0	25		40	96	100	67	93	88	82		38			12	16	
	0	23		66	71	72	66	78	68					25	8	0	
	0	30	20	54	52		100		58	60					24		4
essing	0	13	31	90		60*	74	75	56	82		41		8		27	
KA	0	25	31	83		83	64	100		82		63		22		8	
	0	15	17	70	58	72	72	83		76		36		12	4		4
	0	22	12	43	53	95	69	84		58		20	40		10		
mous	0	8	24	55	62	57	100	92	71	82		57		30	10	25	
	0		36	86	80	66		72	70	43		26		0	12	4	
inoma	0		48	40	80	42		37	100	89		86		7	13		2
	0		25		60			79	82	62		0			0		11

* at 4.5 hours

Figure A1

Means ± Standard Errors of Percent Labelled Mitoses

Versus Time of Telogen IFE

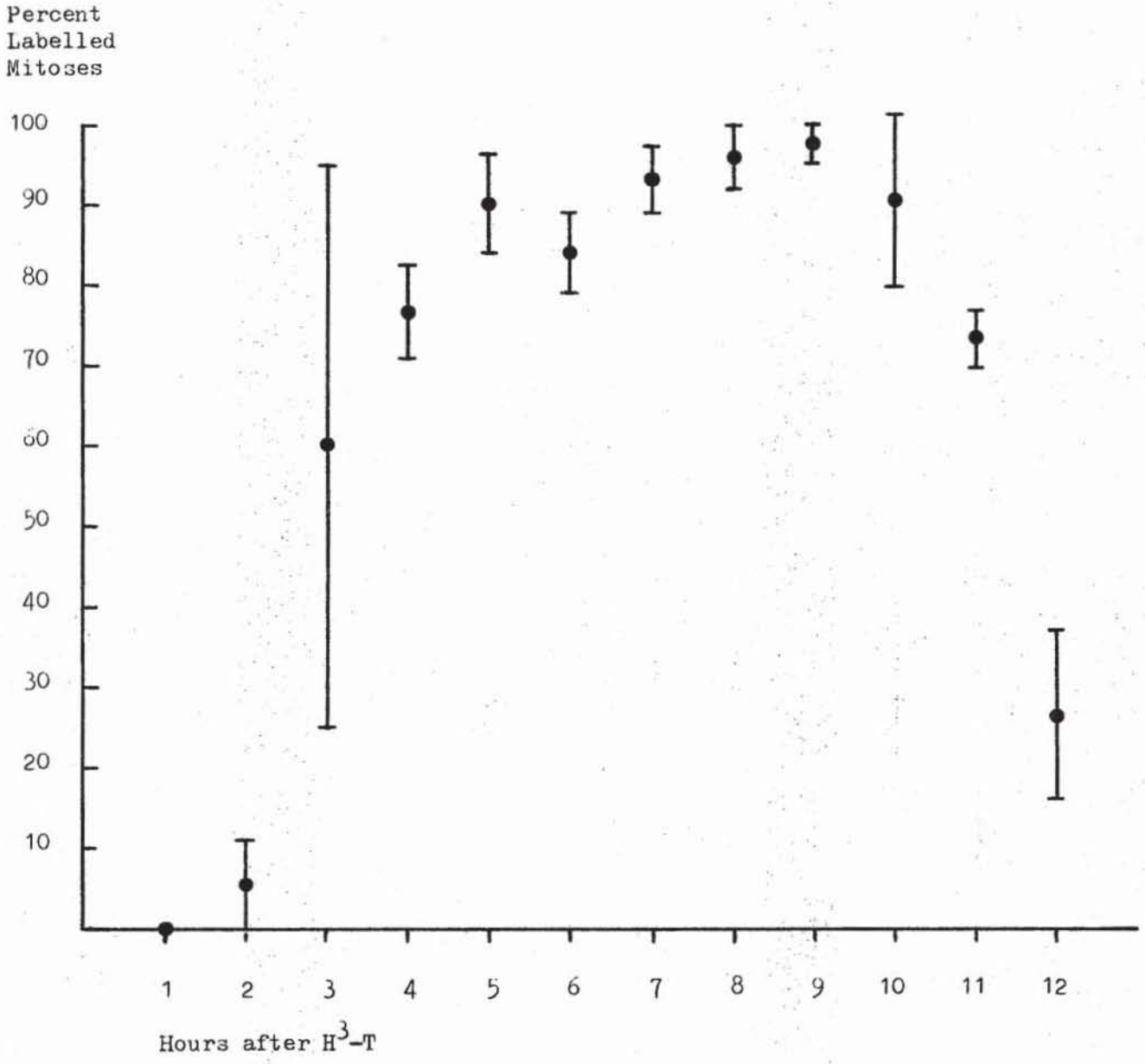


Figure A2

Means ± Standard Errors of Percent Labelled Mitoses

Versus Time of Anagen VI IFE

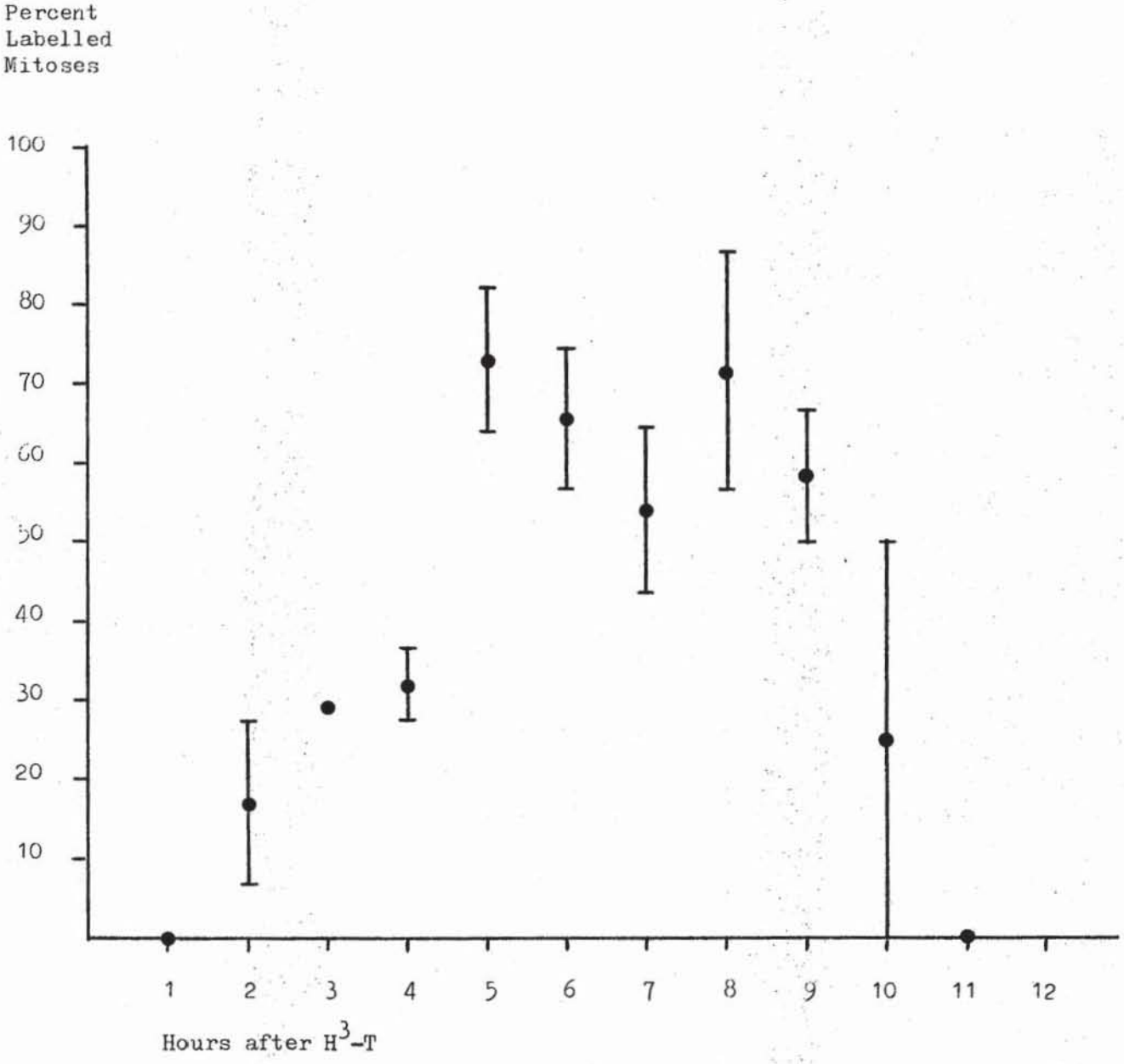
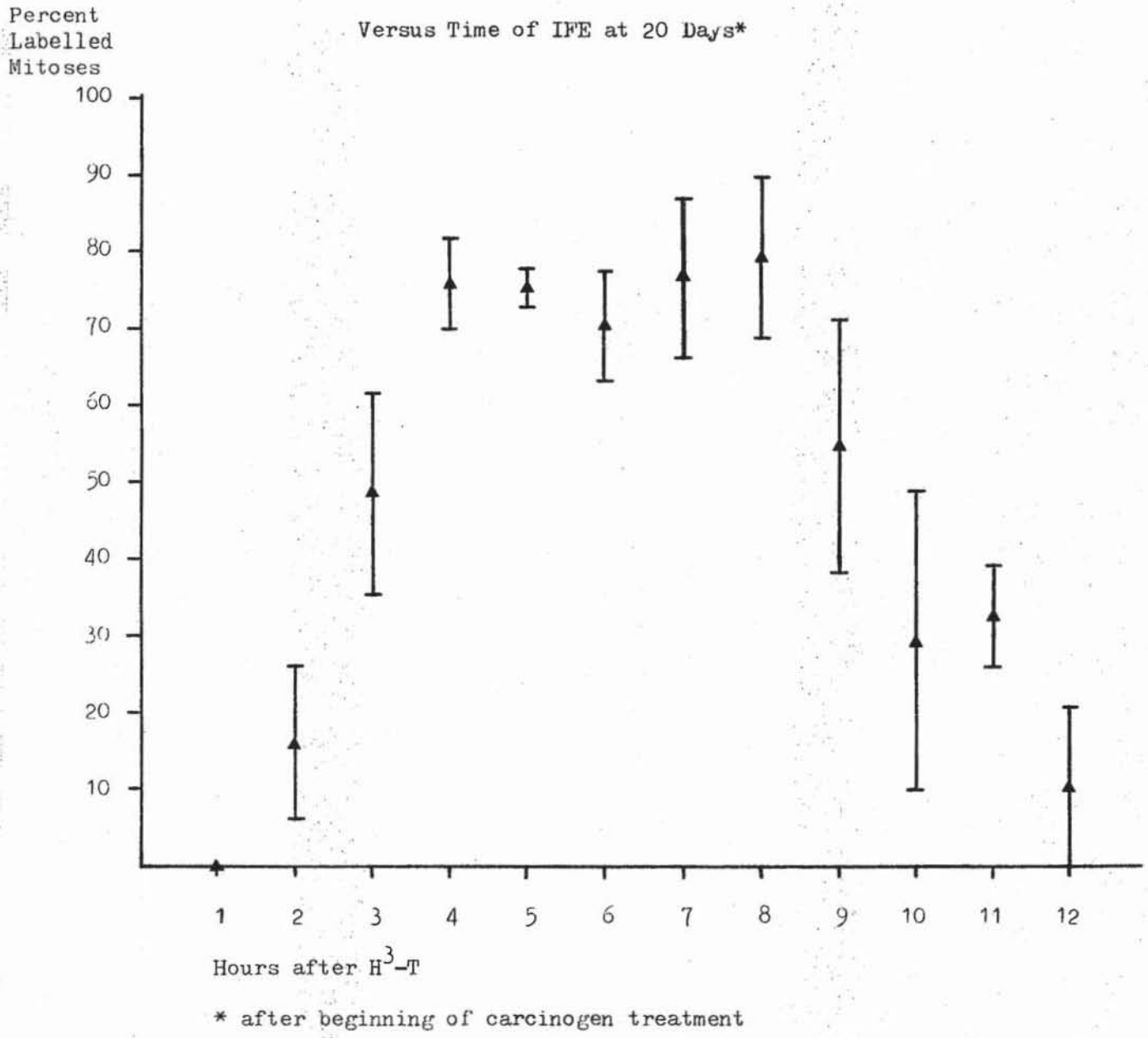


Figure A3

Means ± Standard Errors of Percent Labelled Mitoses

Versus Time of IFE at 20 Days*

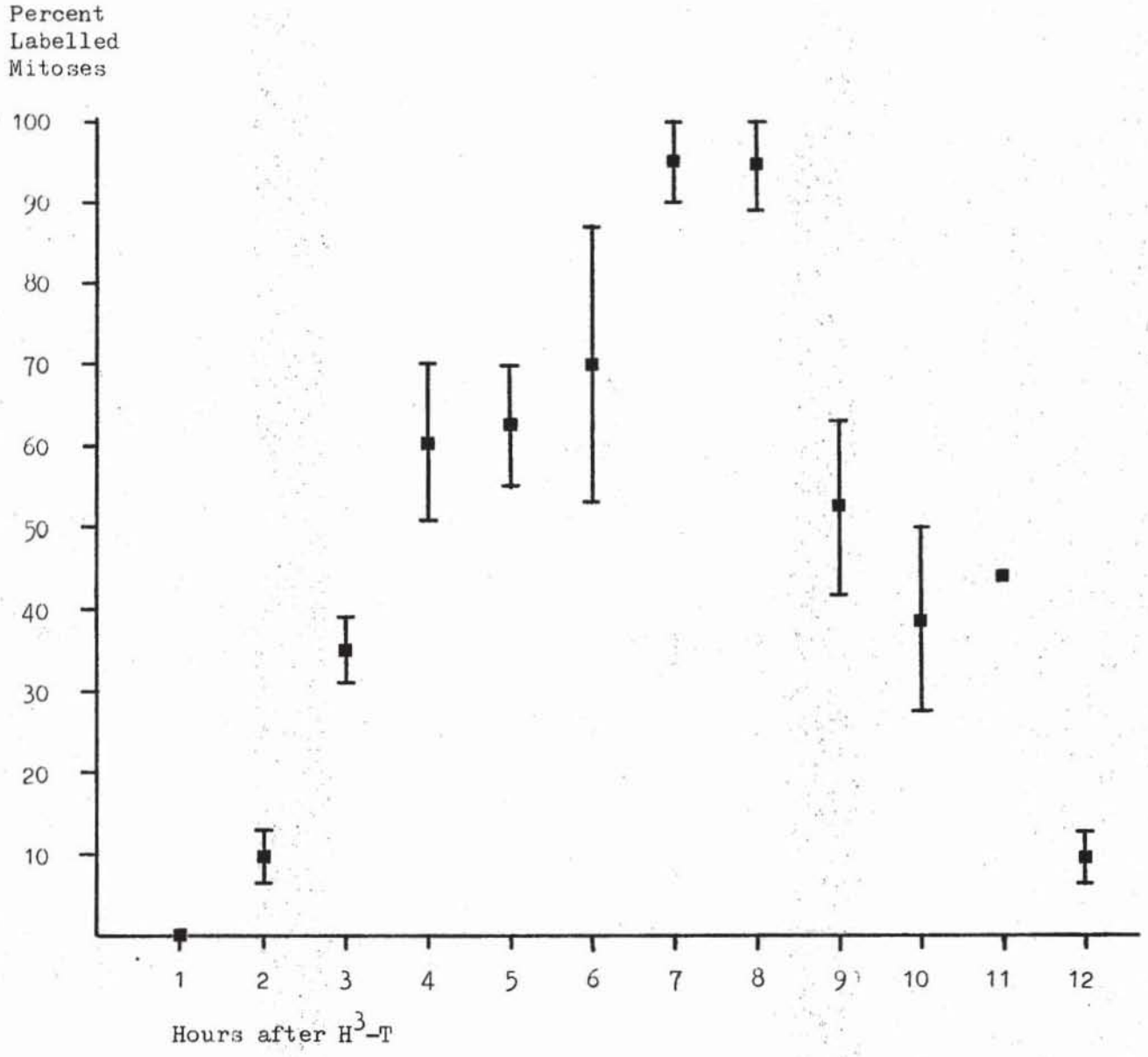


* after beginning of carcinogen treatment

Figure A4

Means ± Standard Errors of Percent Labelled Mitoses

Versus Time of IFE at 40 Days*

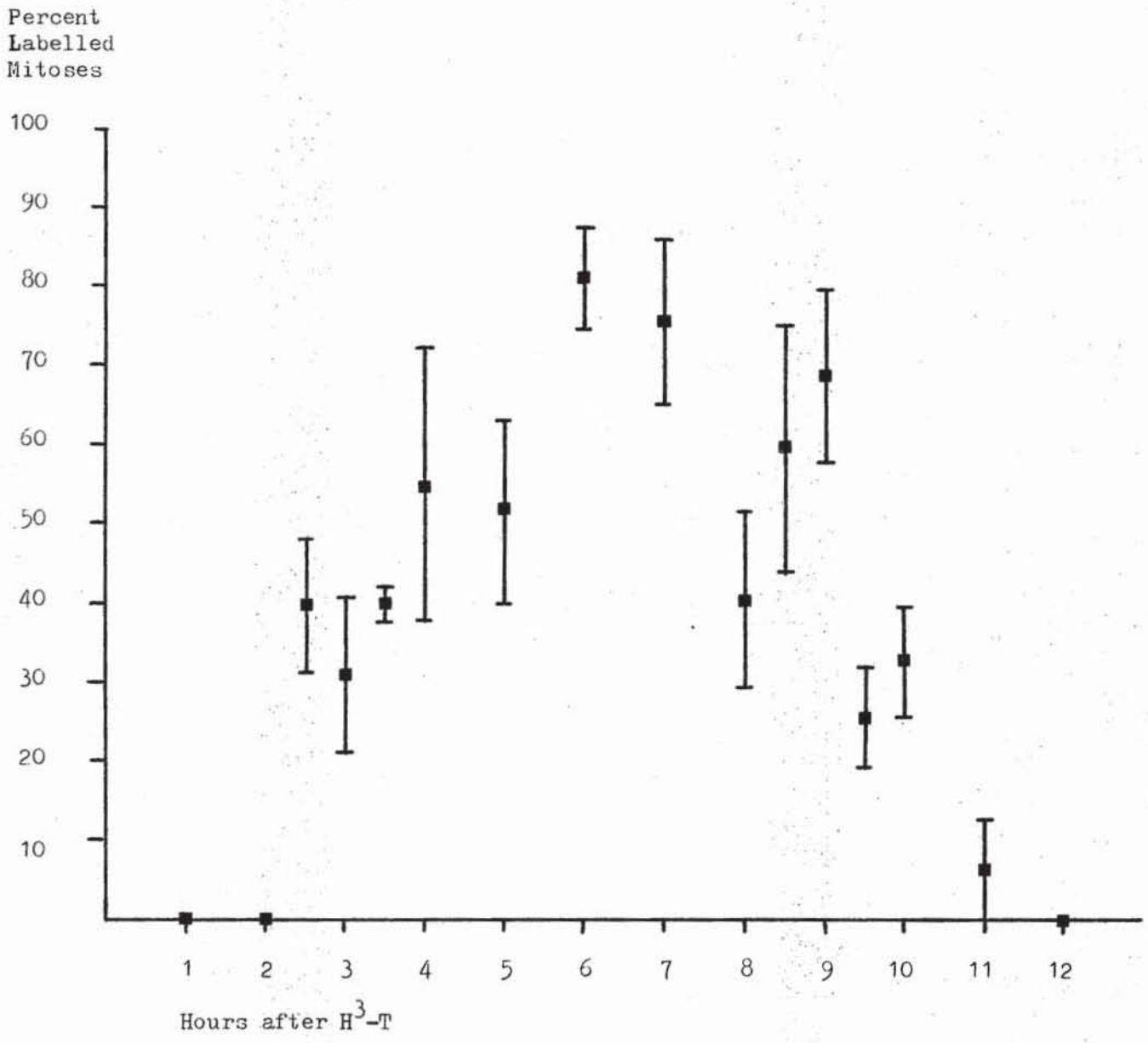


* after beginning of carcinogen treatment

Figure A5

Means ± Standard Errors of Percent Labelled Mitoses

Versus Time of IFE at 60 Days*

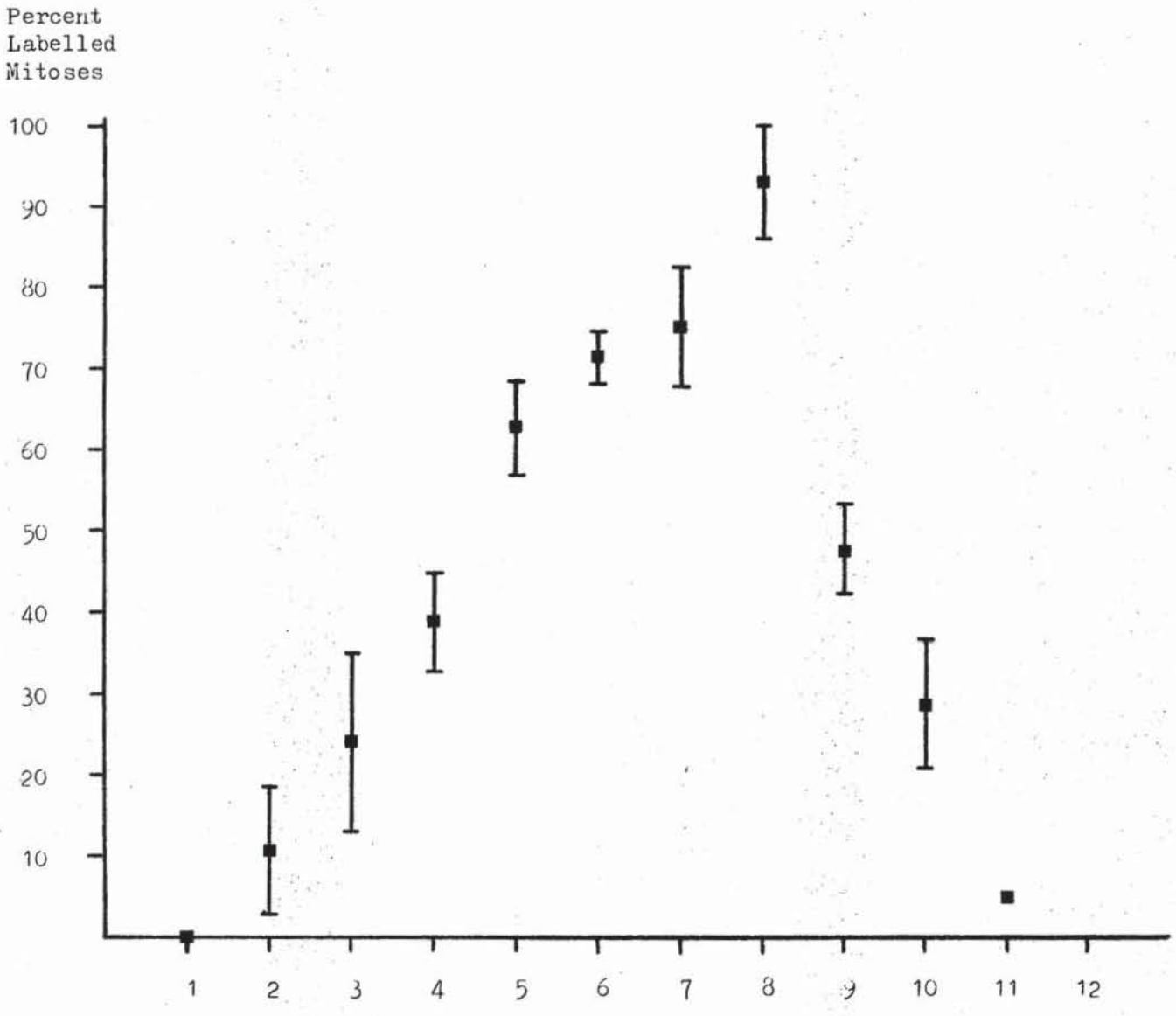


* after beginning of carcinogen treatment

Figure A6

Means ± Standard Errors of Percent Labelled Mitoses

Versus Time of IFE at 80 Days*

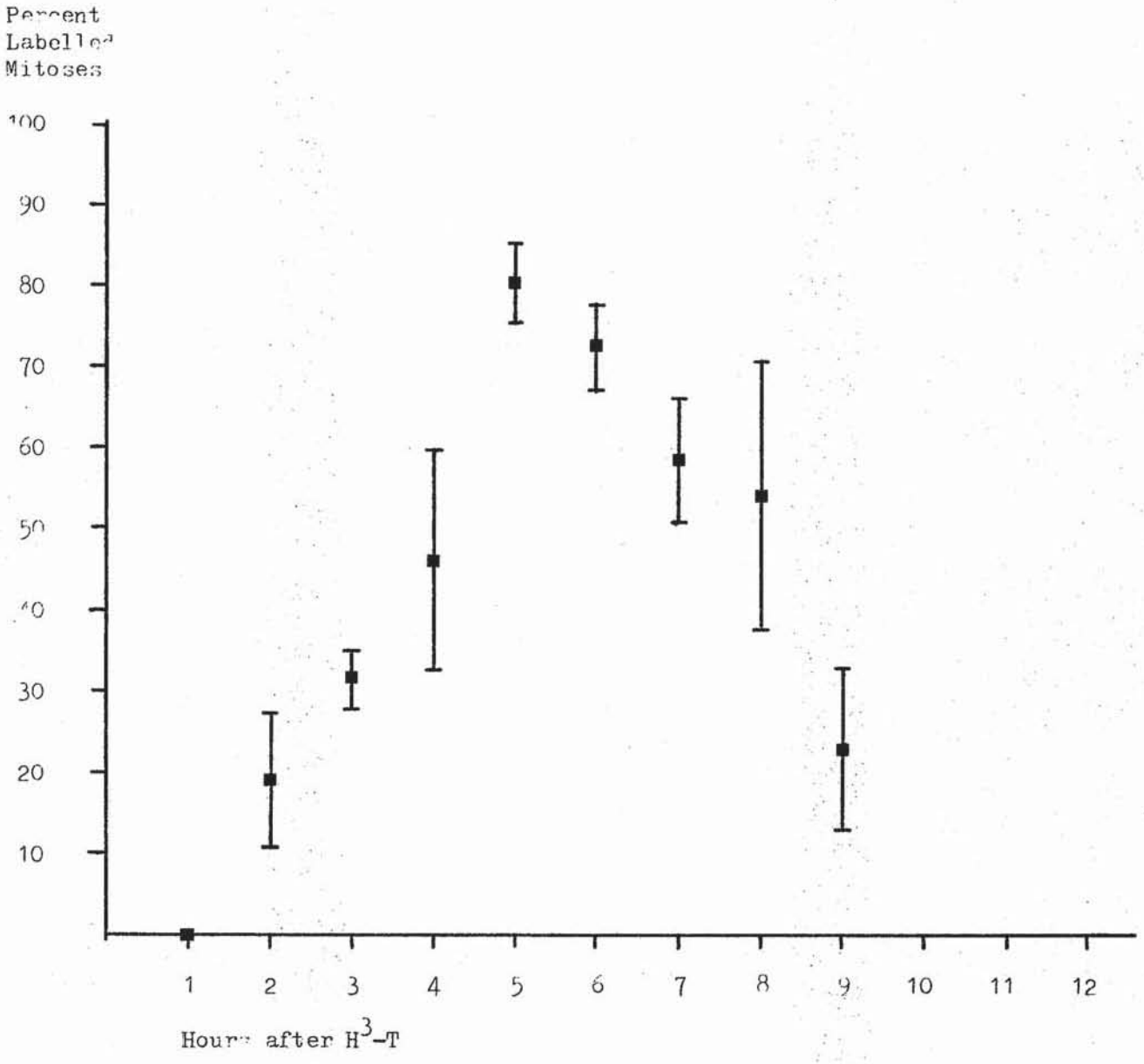


* after beginning of carcinogen treatment

Figure A7

Means ± Standard Errors of Percent Labelled Mitoses

Versus Time of IFE at 100 Days*

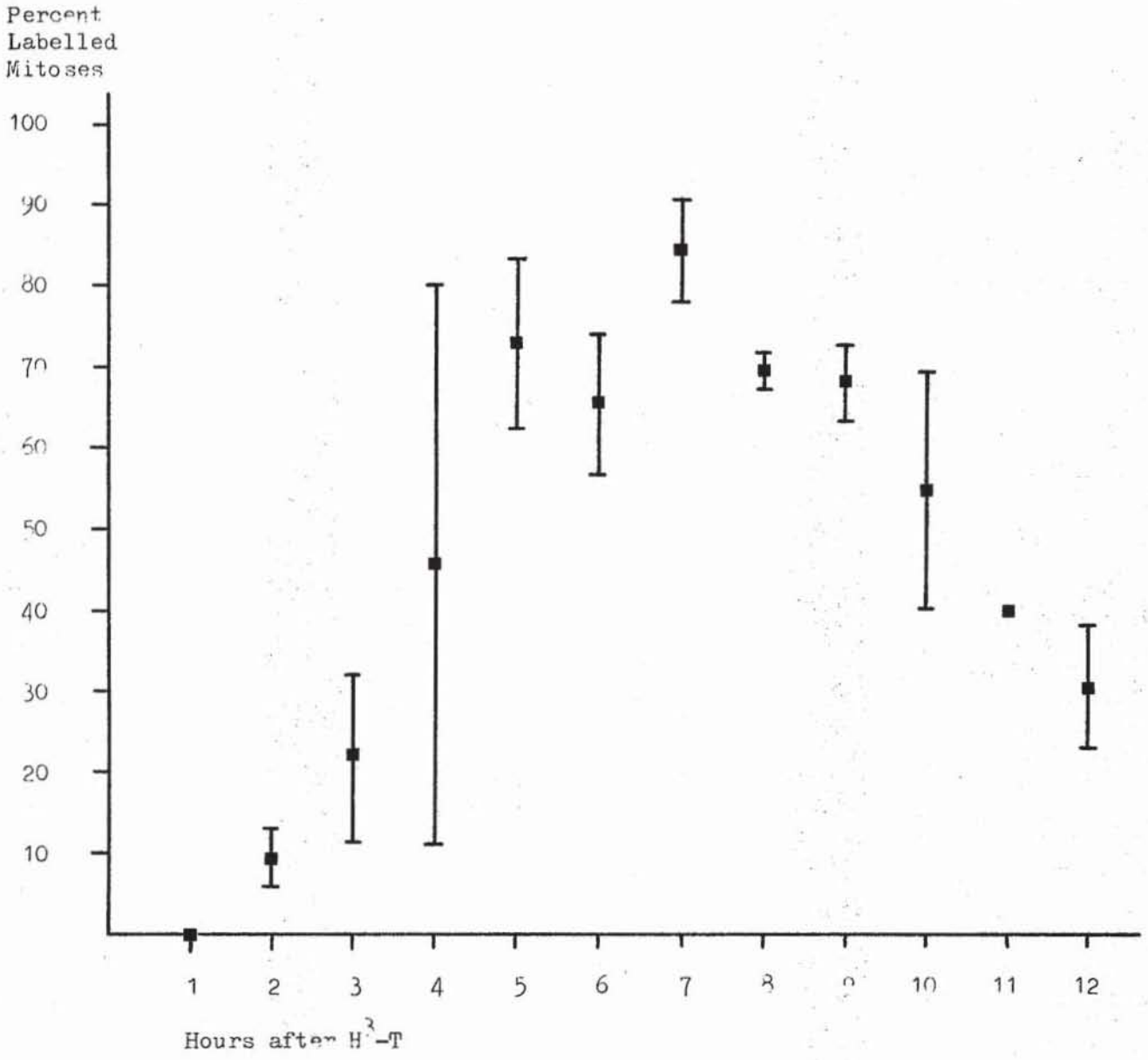


* after beginning of carcinogen treatment

Figure A8

Means ± Standard Errors of Percent Labelled Mitoses

Versus Time of IFE at 120 Days*



* after beginning of carcinogen treatment

Figure A9

Means ± Standard Errors of Percent Labelled Mitoses

Versus Time of Early Keratoacanthoma

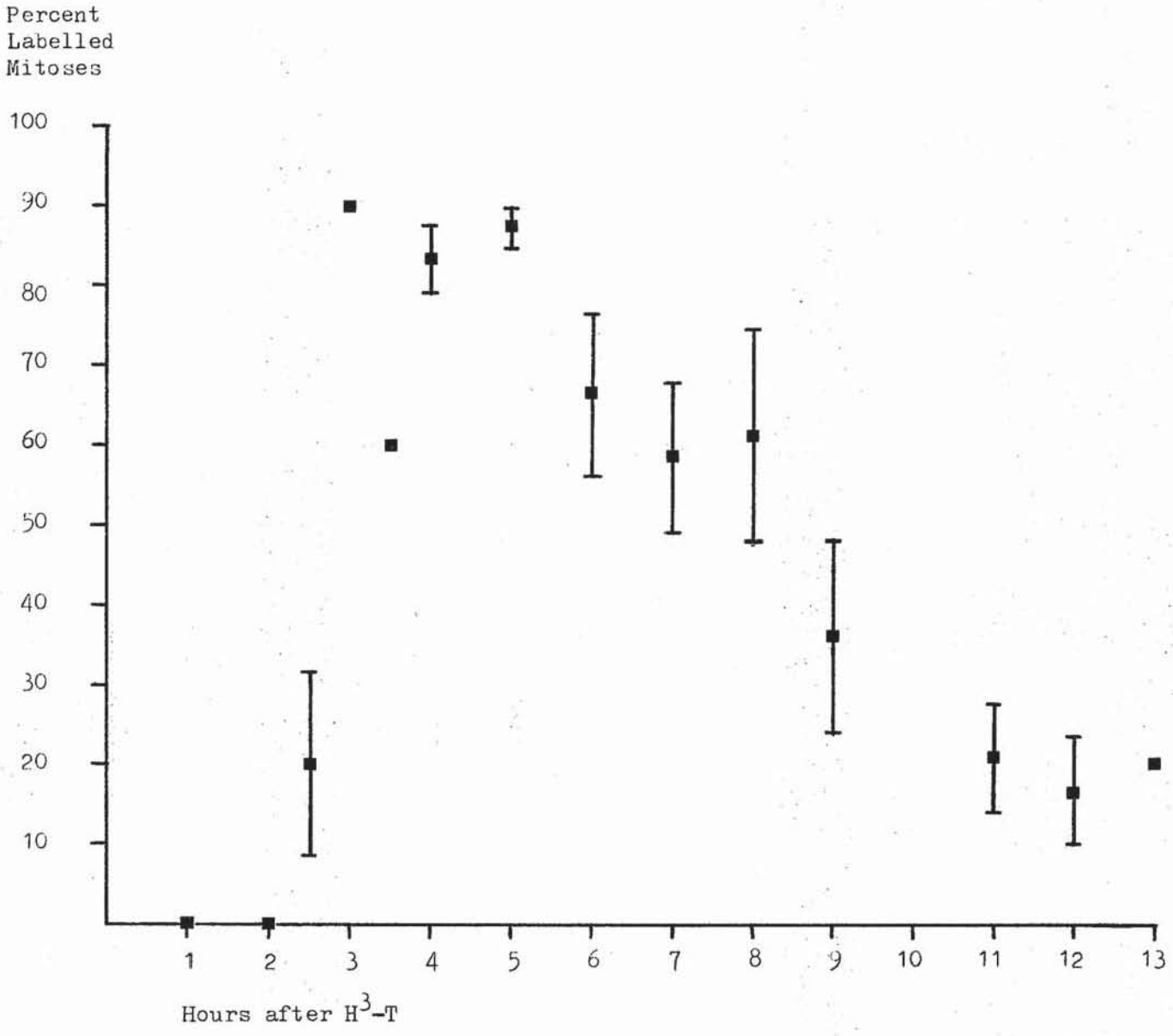


Figure A10

Means ± Standard Errors of Percent Labelled Mitoses

Versus Time of Mature Keratoacanthoma

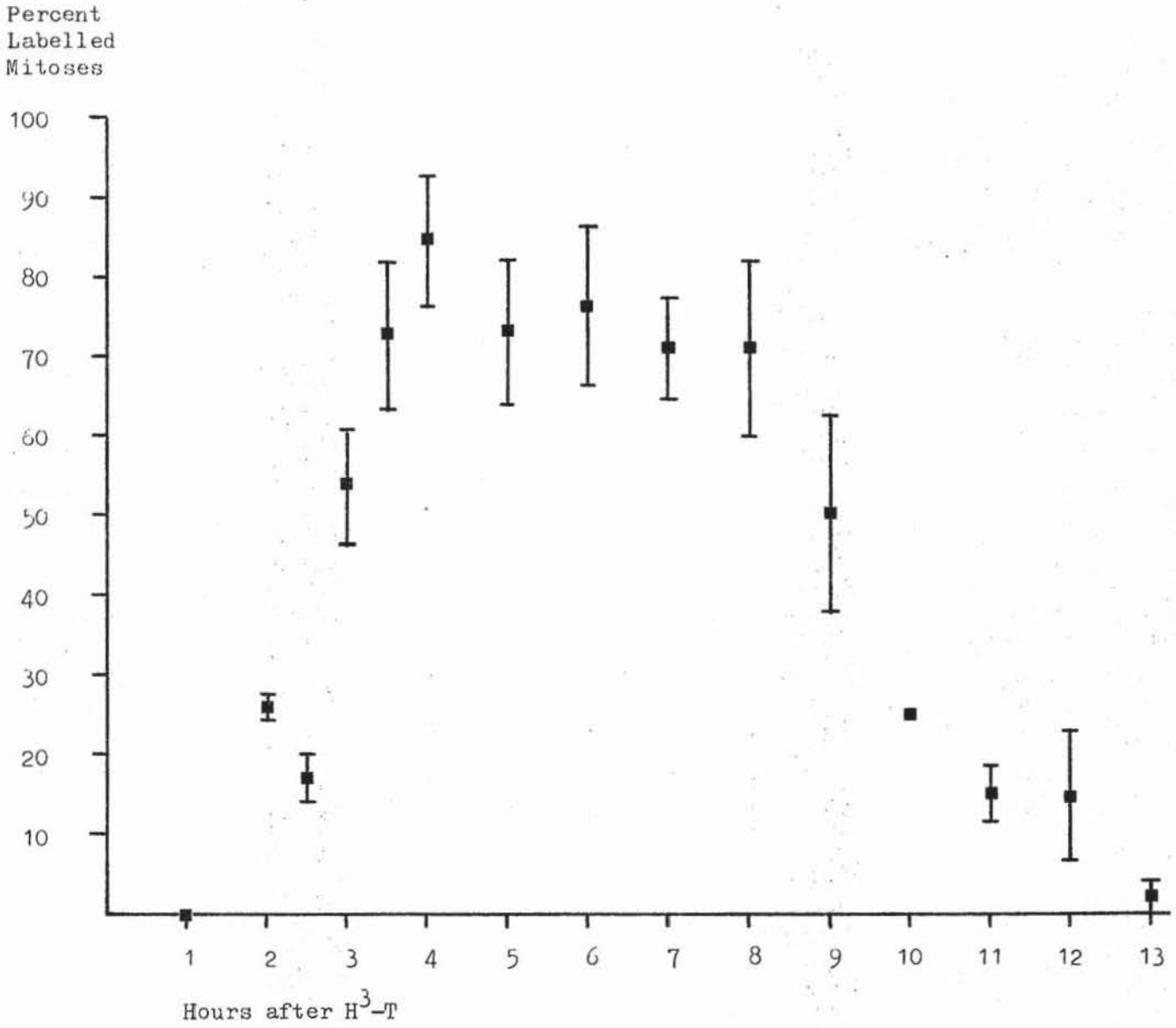


Figure A11

Means ± Standard Errors of Percent Labelled Mitoses

Versus Time of Regressing Keratoacanthoma

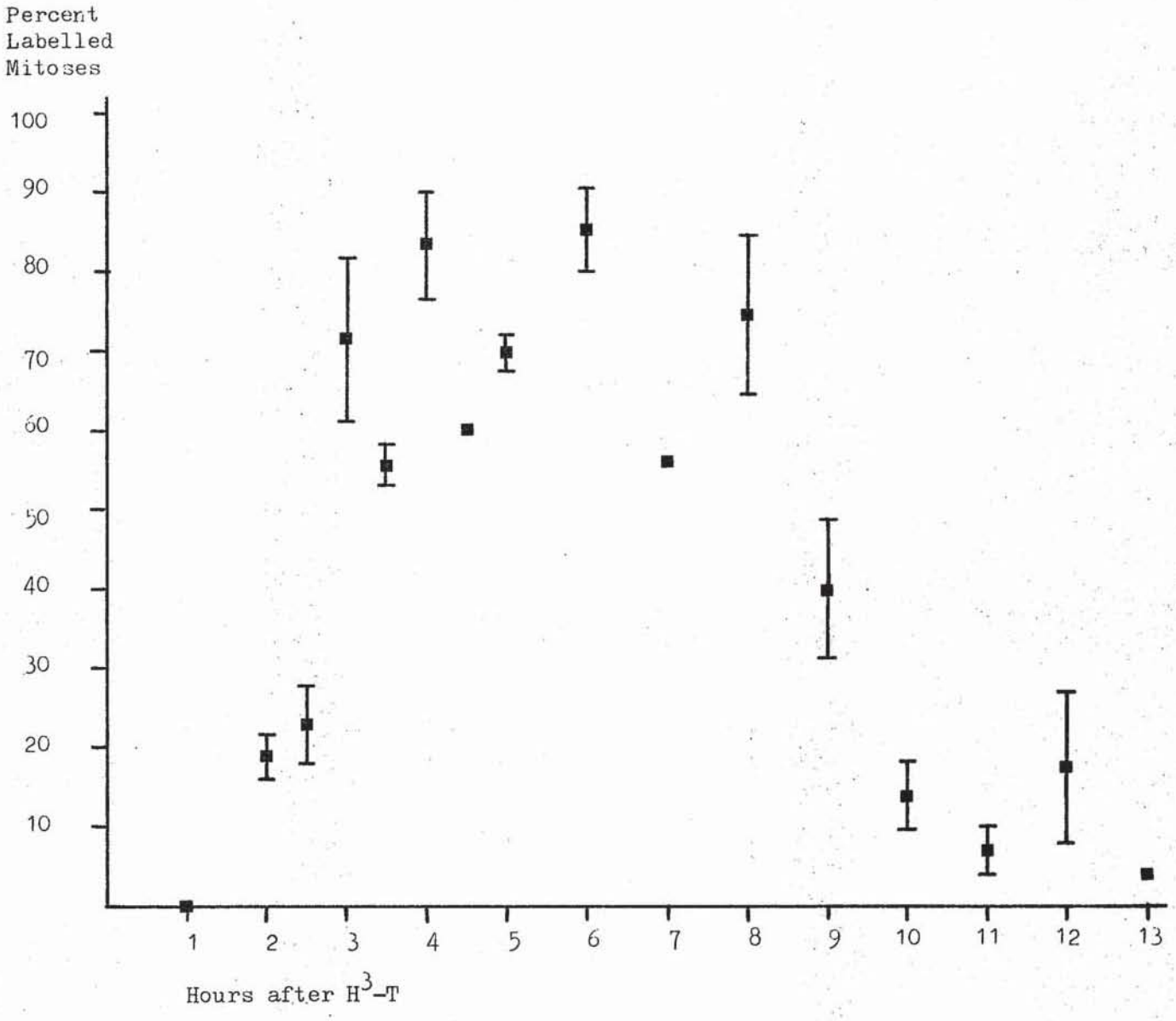


Figure A12

Means ± Standard Errors of Percent Labelled Mitoses

Versus Time of Squamous Cell Carcinoma