THE CELLULAR EFFECTS OF CYTOSINE ARABINOSIDE ON MURINE B16 MELANOMA AND EHRLICH ASCITES TUMOR

A Thesis

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by

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To my wife and parents

to whom I owe so much.

ABSTRACT

Cytosine arabinoside (ara-C), a synthetic nucleoside has been shown to inhibit DNA synthesis. Following treatment with ara-C a reduction was reported in the number of cells undergoing cell division. With this in mind, the purpose of this study was to quantitatively determine the effects of ara-C upon B16 melanoma and Ehrlich ascites tumor (EAT) cell populations. The quantitative parameters observed were the daily mitotic rates, the thymidine index and the durations of the cell cycle phases for both tumor cell populations. The mitotic rates of B16 melanoma and EAT were treated with single and multiple injections of ara-C, with host recovery periods.

Fifty and 80 mg/kg doses of ara-C reduced the daily mitotic rate of B16 melanoma, on day 10 of tumor growth, by 67 and 45 percent respectively. After a 50 mg/kg dose of ara-C the daily mitotic rate of EAT, on day 6 of tumor growth, was reduced by 38 percent.

In both the B16 melanoma and the EAT cells three successive daily injections of ara-C were more effective than either two successive daily injections or one single injection of ara-C. Ara-C had no effect upon the transplantability of either B16 melanoma or EAT cells.

Ara-C (50 mg/kg) blocked the uptake of tritiated thymidine by cells synthesizing DNA, within 15 minutes after being injected. The thymidine index began to recover, suggesting that the effective levels of ara-C were maintained in the tumor bearing mice for about one hour after its administration. Ara-C also synchronized a portion of both the B16 melanoma and the EAT cell populations.

The phases of the cell cycle were reported for the first time for the B16 melanoma, while the cell cycle phases found for the EAT cells agreed with data published by other authors. The cell cycles of both the B16 melanoma and the EAT cells after treatment were delayed by an interval equal to the duration of the DNA-synthesizing (t_S) and pre-mitotic (t_{G2}) phases. This confirms previous findings that ara-C blocks the entry of cells into the S-phase of the cell cycle. Results suggest that the S-phase may be shortened after ara-C treatment in the B16 melanoma.

It was found that four injections of ara-C (50 mg/kg), at eight hour intervals, was too toxic for the B16 melanoma bearing mice. In the same experiment it was found that a host recovery time of three days allowed a complete recovery of the mitotic rate of the B16 melanoma, thus providing no chemotherapeutic advantage. Four injections of ara-C (25 mg/kg), at eight hour intervals, was also toxic but to a lesser degree than the 50 mg/kg dose. This dose regimen of ara-C also reduced the mitotic rate of the EAT cells but there was some recovery. A host recovery time of one day was more suitable as it allowed a smaller degree of mitotic rate recovery in both tumors. The injections of four 12.5 mg/kg doses of ara-C, at eight hour intervals were very ineffective in maintaining a reduced mitotic rate in the B16 melanoma but seemed to synchronize a portion of the cell population. Such a synchronization was shown by an "over-shoot" of the recovering mitotic rates over the mean control values.

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TABLE OF CONTENTS

CHAPTER PAGE

CHAPTER PAGE

LIST OF TABLES

TABLE PAGE

LIST OF FIGURES

FIGURE PAGE

LIST OF PHOTOGRAPHS

CHAPTER I.

INTRODUCTION

1. Problems

Experiment No: M1. The purpose of this experiment was to determine the daily mitotic rates of the B16 melanoma after treating tumor-bearing mice with dosages of 50 and 80 mg/kg of ara-C on the 10th day of tumor transplantation. An equally important part of this experiment was to determine which of the two doses was more effective.

Experiment No: M2. The purpose of this experiment was to determine the effect of two and three serial injections of ara-C, given at daily intervals, upon the mitotic rate of the B16 melanoma.

Experiment No: M3. The purpose of this experiment was to ascertain whether ara-C affected the transplantability of B16 melanoma tumor cells.

Experiment No: M4. The aim of this experiment was to determine the effect of a single 50 mg/kg dose of ara-C upon the thymidine index of the B16 melanoma tumor cells.

Experiment No: M5. The purpose of this experiment was to determine the effect of ara-C (50 mg/kg) treatment upon the cell cycle of B16 melanoma tumor cells.

Experiment No: M6. The aim of this experiment was to ascertain the effect on the mitotic activity of B16 melanoma of a 3 day host rest period allowed between two series of ara-C injections (50 mg/kg) on days 6 and 10 after tumor transplantation.

Experiment No: M7. The purpose of this experiment was to ascertain the effect on the mitotic activity of B16 melanoma tumors of four intraperitoneal injections of ara-C (25 mg/kg) at eight hour intervals on

days 6, 8 and 10 after tumor transplantation. The host recovery period was reduced to one day intervening between the series of injections.

Experiment No: M8. The aim of this experiment was to ascertain the mitotic activity of B16 melanoma tumors from mice receiving four injections of ara-C (12.5 mg/kg) at eight hour intervals on days 6, 8 and 10 after tumor transplantation. The host recovery time was one day intervening between the series of injections.

Experiment No: A1. The purpose of this experiment was to determine the daily mitotic rate of Ehrlich ascites tumor cells (EAT) after treating tumor-bearing mice with a single (50 mg/kg) dose of ara-C.

Experiment No: A2. The purpose of this experiment was to determine the effect of two and three serial injections of ara-C, given at daily intervals, upon the mitotic rate of EAT cells.

Experiment No: A3. The aim of this experiment was to determine whether a single 50 mg/kg dose of ara-C affected the transplantation of EAT cells.

Experiment No: A4. The aim of this experiment was to determine the effect of a single 50 mg/kg dose of ara-C upon the thymidine index of the EAT cells.

Experiment No: A5. The purpose of this experiment was to determine the effect of ara-C (50 mg/kg) treatment upon the cell cycle of EAT cells.

Experiment No: A6. The purpose of this experiment was to ascertain the mitotic activity of EAT cells from mice receiving four injections of ara-C (25 mg/kg) at eight hour intervals on days 3, 5 and 7 after tumor

transplantation, with one day intervening between the series of injections.

2. The Importance of the Study.

Since the advent of chemotherapeutic treatment for malignant neoplasms various drugs have been used to attempt a complete cure. The above mentioned experiments are preliminary studies on the effects of ara-C upon the mitotic activity of malignant cell proliferation. Other workers have studied the effects of various dose regimen of ara-C upon the survival times of tumor-bearing mice (Skipper et al., 1965, 1967), and *in vitro* studies on the cell cycle (Brehaut and Fitzgerald, 1968; and Karon and Shirakawa, 1969); but although the effect of ara-C as a mitotic inhibitor has been mentioned (Heneen and Nichols, 1967), no quantitative *in vivo* study has been undertaken.

The results of this work will serve as a basis for further studies on how to attack malignant cell proliferation both by ara-C and possibly other drugs. If a complete inhibition of mitotic activity can be maintained for an extended period of time by ara-C this may serve to kill the tumor's proliferating population. If, on the other hand, after a period of quiescence there is a sudden burst of mitotic activity, then there may be a G_o phase present. A cell in the G_o phase is one which does not undergo DNA synthesis, during a dormant phase, and consequently will not be affected by ara-C treatment. Such a cell is not in the continuous cell cycle and remains dormant until such a time as it is triggered into the cell cycle. What triggers this, or even whether such a G_o cell exists is at present unknown. One possibility is that a small portion

of a tumor population may be in an extended G_1 phase thus acting as the described G_o. This is one of the questions which will be looked into in future experiments.

The knowledge that can be gained about abnormal cell proliferation and methods which can be used to combat this can surely be used to advantage in the battle against tumor growth.

CHAPTER II.

REVIEW OF THE LITERATURE

GROWTH AND CELL PROLIFERATION

Growth can be arbitrarily divided into two main types, normal and abnormal. Dorland (1965) describes normal growth as a process of increase in size, produced by accretion of tissue of a constitution, similar to that originally present. This type of growth is best illustrated by the development of a fetus to a child and eventually an adult. Growth in this case is due to an actual increase in total cell number arising by mitosis. Such growth is controlled by the intricate interactions of tissue environment or milieu and usually has no deleterious effect upon the body.

Abnormal growth necessarily has the opposite characteristics of normally growing tissue. It is generally supposed that abnormal growth is brought about by a loss of some controlling factor(s), allowing the tissue to grow indiscriminately and often with unlimited bounds. Tumors fit into this simple classification of abnormal tissue growth. Willis (1960) defined a tumor thusly,

> "A tumor is an abnormal mass of tissue, the growth of which exceeds and is uncoordinated with that of the normal tissues, and persists in the same excessive manner after cessation of the stimuli which evoke the change."

This indiscriminate growth of tissue may or may not be harmful to the host. Tumors that are called "innocent" or benign grow rather slowly and contain few mitotic figures at any instant of time. Benign tumors seldom kill the host and usually remain encapsulated. In contrast, malignant tumors grow rapidly and usually contain numerous mitotic figures. Such tumors always cease to grow only on the death of the host. Willis (1960) describes a malignant tumor as:

> "Tumors of the same cell-type grow rapidly, invade neighbouring tissues, spread by metastasis, and unless extirpated at an early stage, inevitably prove fatal."

Growth having been explained in general terms, will now be examined more closely. Generally speaking, there are three types of growing or proliferating cell populations: (1) a population increasing in size caused by cell addition exceeding cell loss, e.g. embryonic tissues or tissue cultures. (2) A steady-state renewal population, where cell formation equals cell loss, as in epidermis or intestinal epithelium. (3) A population which decreases in size as witnessed in a damaged population (Wimber, 1963) or in atrophy, where cell loss is greater than cell production. In adult tissues the most common type of proliferating cell population is the so-called "steady-state cell population". One criterion used to establish whether a tissue is renewing and also its degree of renewal, is the visual presence of mitotic figures. As Leblond and Walker (1956) explained, the presence of mitotic figures in some tissues are readily understood in growing areas such as hair or ovarian follicles, but seem less plausible in tissues without a change in cell number or size. This lack of change in both cell number and overall size has been theoretically explained by a steady-state system, where cell production equals cell loss; and it can be seen in fact in some body tissues, e.g. the intestinal mucosa, where these two parameters are equal.

Mitotic activity can be estimated for any tissue by ascertaining its mitotic index the simplest estimation of mitotic activity that can be made. The mitotic index is the number of dividing cells per unit cell population

(usually expressed as per 1000 cells). This figure alone will give some indication of mitotic activity if compared to mitotic indices of other tissues. To determine the mitotic rates of more slowly proliferating tissues, additional aids, such as colchicine arrest of mitosis, must be used.

The colchicine technique employs the drug colchicine, a plant alkaloid, which has the property of preventing cell division proceeding beyond metaphase. If the proper conditions using colchicine are met, a great deal of information on the mitotic activity of a tissue can be gleaned. Stevens-Hooper (1961) concluded that "mitosis is neither stimulated nor inhibited since the presence of colchicine did not change the normal rate of initiating prophases, nor its duration". Complete colchicine arrest is achieved in normal mouse tissues at a dosage of 0.20 mg. per 100 gm. body weight (Bertalanffy and Leblond, 1953); thus the same dosage is also optimal for neoplastic cell populations in the mouse (Wallace, 1964). The interval of colchicine action usually applied for mouse and rat tissues is four to six hours (Bertalanffy and Lau, 1962). After this time interval a disintegration of colchicine metaphases arrested early after the action of the drug may occur, which would lower the estimation of mitotic activity. Colchicine usually acts after a 15 - 30 minute delay following its subcutaneous injection. Such a lag was shown in Ehrlich ascites tumor cells by Bertalanffy et al. (1965). It was also demonstrated by Bertalanffy and Leblond (1953), and Stevens-Hooper (1961) that colchicine worked uniformly over a six hour period, thus giving a complete block of mitosis during this period. Colchicine has without doubt been a useful tool for the evaluation of mitotic activity for many years.

The mitotic rate, that is, the rate of accumulation of blocked metaphases (or the time required for a given percentage of cells to undergo mitosis), can be determined by the colchicine technique. The percentage of metaphases accumulated four hours after the injection of colchicine yields a mitotic rate for this four hour period. If six groups of animals are injected with colchicine at four hour intervals over a 24-hour period, summation of the six individual 4-hour percentages yields the total percentage of cells entering mitosis during the 24-hour period, that is, the daily mitotic rate. Once this percentage is known for 24 hours then the turnover or renewal time, equal to the time for the replacement of 100 percent of the cells, can be calculated (Leblond and Walker, 1956; and Stevens-Hooper, 1961).

The duration of mitosis can be calculated by using a combination of the colchicine technique and the percentage of nuclei in the various stages of mitosis (prophase, metaphase, anaphase and telophase), by the formula

$$\text{Duration of Stage x} = \frac{\%\text{ nuclei at stage x in normal tissue}}{\%\text{ nuclei in metaphase after t. min. colchicine}} \times \text{t. min. of colchicine treatment}$$

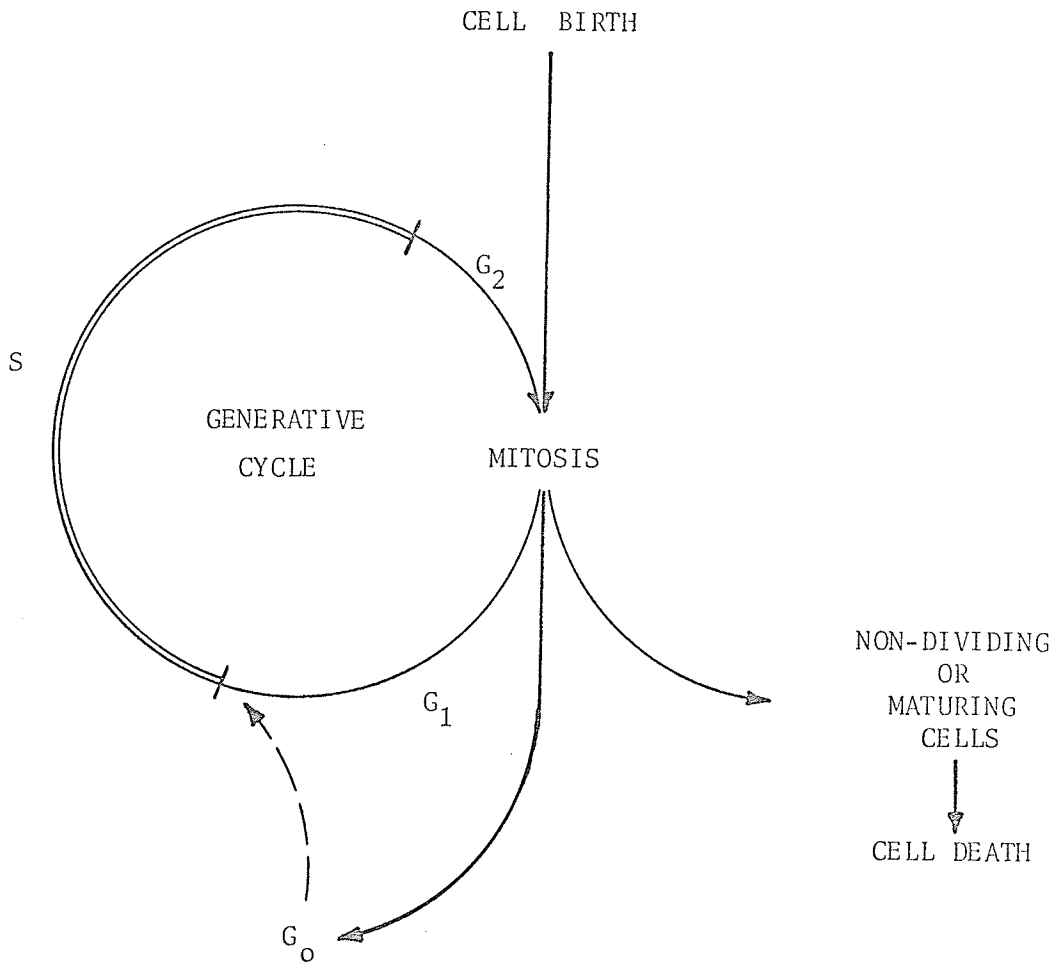
developed by Leblond and Stevens (1948) and Stevens-Hooper (1961). Although precise data on the rate of cell production are obtained by the colchicine technique, one shortcoming is that the migration of cells through a tissue cannot be followed with this technique. Recently DNA-labelling compounds, such as tritiated thymidine, have been employed to obtain the latter information, and to explore the cell cycle and turnover times of both normal and tumorous tissues.

Tritiated thymidine combined with high resolution autoradiography provided a clearer understanding of the phases of the cell cycle. Baserga (1965) defined the cell cycle as the orderly sequence of metabolic activities, from the midpoint of mitosis to the midpoint of a successive mitosis. The cell cycle (Figure 1) was initially described by Howard and Pelc (1951) using isotope P^{32} labeling, while it was merely divided into the mitotic phase and interphase. Mitosis is that stage which is morphologically different from interphase and results in the division of the chromosomes and the subsequent formation of two daughter cells. The morphological stages of mitosis have been described in detail by Stevens-Hooper (1961), and are outlined in the chapter dealing with materials and methods. The duration of mitosis ranges from 30 minutes to 2.5 hours in different cell populations. During mitosis, RNA synthesis ceases and protein synthesis decreases (Baserga, 1968).

Howard and Pelc (1951) further subdivided interphase into three phases which appear by experimental evidence to be separate but in fact may blend one into the other. The post-mitotic phase (G_1) is the mystery stage of the cell cycle, as little is yet known of the biochemical events that occur. It is thought that both protein and RNA synthesis take place and that these are required for an even flow from G_1 to the DNA synthesis stage (Baserga, 1968). It has been shown that G_1 phase of the cycle is the most variable in length, ranging in proliferating cell populations from zero in Ehrlich ascites tumor cells (Baserga, 1963) to an estimated 123.5 hours in the cheek pouch epithelium of hamsters (Brown and Oliver, 1968).

FIGURE 1

THE CELL CYCLE



Modified from
Quastler (1963).

The post-mitotic phase flows gradually into the DNA synthesizing (S) phase of the cell cycle. The latter is thought to be the only stage of the cycle when DNA is synthesized; although it is not the only anabolic activity occurring during that period. RNA synthesis has been shown to continue during this time (Baserga, 1962a), and the rate of protein synthesis increases in mammalian cells as shown by Baserga (1962b), and Baserga and Kisieleski (1962). With the DNA compliment of a diploid cell doubled, the cell progresses into the pre-mitotic or G_2 phase, when the cells continue to synthesize RNA and proteins, but not DNA. If protein synthesis is inhibited with puromycin, or RNA synthesis inhibited by actinomycin, mitosis is delayed (Donnelly and Sisken, 1967).

Gelfant (1962) suggested that in mouse epidermis there are cells delayed in G_2 for long periods of time. These cells are thus ready to divide within a relatively short period of time. Thus M can be initiated from the G_2 phase quickly without undergoing further DNA synthesis. The length of the G_2 phase is perhaps the most constant, lasting from about 1-1/2 to 2-1/2 hours, although some variations were reported by Baserga (1965).

Recently, another concept has been added to the cell cycle, that of the G_o phase, or true resting stage (Quastler, 1963; and Lajtha, 1963). The cells of the G_o fraction are potentially proliferative, and appear morphologically identical to a cell in G_1; yet, they do not undergo differential growth or DNA synthesis, or only at a very slow rate. As Lajtha (1963) expressed it "they just sit there minding their own biochemical business". The G_o fraction is not part of the cell cycle itself but cells

of this fraction can be triggered into the cell cycle should the need arise. Once the cells, either in the G_1 or G_o fraction, are triggered into DNA synthesis, they are obligated to complete mitosis after passing through G_2. It is thought that control of DNA synthesis and thus division, is initiated somewhere in the G_1 phase of the cell cycle. Prescott (1964) stated that adequate nucleotide pools, DNA polymerase(s), and DNA activation are all necessary for the initiation of DNA synthesis, but in some way these factors seem not sufficient for the biological regulations of DNA synthesis.

Determination of the Cell Cycle. The most common label used for the determination of the cell cycle has been tritium labeled thymidine or tritiated thymidine ($TdR\text{-}H^3$). Tritiated thymidine, an artificial precursor of DNA was first shown to be incorporated exclusively into mammalian cells undergoing DNA synthesis (Hughes et al, 1958). These authors also assumed that $TdR\text{-}H^3$ does not label cells undergoing DNA synthesis longer than one hour after injection at the most. Blenkinsopp (1968) more recently provided direct proof to this assumption.

The uptake of tritiated thymidine by organs and tissues of mice varied with the route of administration (Petersen and Baserga, 1964). Hinrichs et al (1964) demonstrated that only 8 - 9 % of intraperitoneally injected $TdR\text{-}H^3$ in doses ranging from 10 μC to 100 μC per adult mouse was incorporated into DNA. The remaining $TdR\text{-}H^3$ was quickly catabolized, primarily to tritiated water. A note of caution was expressed by Barr (1963), who observed that exogenous thymidine may have some effect upon

the mitotic cycle. He produced evidence that at some concentrations of thymidine, whether radioactive labelled or non-radioactive, inhibited the mitotic cycle while other concentrations accelerated it. The application of TdR-H^3 as a tool for investigating cell cycles and cell proliferation is greatly aided by two important factors: 1) once a cell has incorporated the label this is retained and 2) the thymidine label becomes diluted only with subsequent cell divisions. Two excellent reviews on the subject of cell proliferation using DNA labels were provided by Wimber (1963) and Lajtha and Gilbert (1967).

TdR-H^3 is most widely used in proliferating cell populations for studies of the cell cycle, first described by Howard and Pelc (1951). The essence of the procedure is to inject TdR-H^3 into a group of mice and kill them at different intervals. Those cells undergoing DNA synthesis at the time the TdR-H^3 was available become labeled, and are demonstrated by the autoradiographic method. The labeled and unlabeled cells are discernable in these autoradiographs. Autoradiographs of tissues collected soon after TdR-H^3 administration display only labeled interphase cells; as time passes labeled mitoses appear. The percentage of the labeled mitoses from individual animals are plotted as a curve against time after the injection of TdR-H^3.

To begin with, only labeled interphase cells are present in the early groups, as those cells were in the S-phase during the injection of TdR-H^3. At a somewhat later period (depending on the duration of the G_2-phase), the first labeled mitoses appear. This time interval, from the injection

of TdR-H^3 to the first appearance of labeled mitoses (prophases), represents the minimal time for the duration of G_2. Once the percentage of labeled mitoses attains 50 percent, this time interval equals $tG_2+1/2tM$. Soon after, the percentage of labeled mitoses will have approached 100 percent. The percentage of mitoses begins subsequently to decline, because the mitoses now appearing were not in the S-phase when TdR-H^3 was available. If these percentages of labeled cells are plotted, the interval between the 50 percent labeled mitoses in the ascending and descending limbs of the first curve equals the duration of the S-phase. After an interval the percentage of mitoses augments once more, constituting the ascending limb of a second curve. This increase is brought about by the labeled daughter cells now undergoing mitosis. The second peak rarely reaches the height of the first curve, partly accountable to errors in detection of labeled cells. The total cell cycle time (t_c) can be estimated by taking a suitable point on the first curve and the identical positional point on the second curve. The time interval between these two points equals t_c. From these percentage labeled mitoses curves the following times can be ascertained: total cell cycle time (t_c), S-phase, and $G_2+1/2M$. The mitotic duration can be calculated by another procedure using colchicine from the mitotic index and the mitotic rate (Leblond and Stevens, 1948; and Stevens-Hooper, 1961). Knowing these data the duration of the G_1- phase can be determined as follows: $G_1 = t_c - (S + G_2 + M)$.

This technique has been most commonly employed and serves as a basis for other procedures such as constant infusion or injection of TdR-H^3

(Mendelsohn, 1962; and Foot, 1963). Moreover, two isotopes have been used simultaneously by Baserga and Lisko (1963), Pilgrim and Maurer (1962), Wimber (1963), and Lala (1958). Further, various authors used a combination of colchicine and TdR-H^3 to estimate the duration of the phases of the cell cycle (Puck and Steffen, 1963; and Maekawa and Tsuchiga, 1968). Such methods are not used widely and often their basis is unfounded.

Another useful bit of information that can be obtained from the use of a radioactive isotope, in particular TdR-H^3, is the thymidine index. The thymidine index, also called the labeling index (Johnson et al, 1960) or the radioactive index (Messier and Leblond, 1960), is the percentage of cells in a given cell population labeled within a short interval after single injection of TdR-H^3 (Baserga and Kisieleski, 1962). This simple information, analogous to the mitotic index, reveals the number of cells undergoing DNA synthesis during the period of availability of TdR-H^3 (Johnson, 1961). The available time for the uptake of TdR-H^3 by cells is usually 30 to 60 minutes. This index gives a figure which can be used to compare the growth rate of both normal and tumorous cell populations (Baserga and Kisieleski, 1962).

Both these methods of determining the: 1) percent labeled mitoses and 2) thymidine index are used in this thesis, and the data are presented in the chapter "Results".

Tumor Growth: Before discussing the growth of Ehrlich ascites tumor cells and mouse B16 melanoma, general comments on tumor growth are in order.

The net growth rate of a tumor is the resultant of cell production and cell loss. Most tumors, whether as solid or ascitic forms, usually increase in cell number, at first at a fairly rapid rate, which with time may gradually decline (Laird, 1964, 1965; and McCredie et al, 1965). The exponential type of equation is the simplest to describe both Krebs (Patt and Blackford 1954) and Ehrlich ascites tumor growth (Baserga, 1964). However, the growth curve of these tumors follows the exponential curve only for a short time, as it falls off later. Another curve applicable to many solid tumors is the Gompertzian function. It describes a cell population whose cells can be regarded as multiplying exponentially but their net accumulation is subjected to some retarding factor(s), whose effect increases exponentially during growth (Laird, 1964 and 1965). Yet, the growth of not all tumors fits either of the above curves, as demonstrated by Frindel et al (1967) with an experimental fibrosarcoma. It became fairly obvious that an appreciable period of tumor growth does not exist having a constant specific growth rate. The trend clearly shows that most tumors grow progressively slower as the tumor increases in size.

As tumor growth is dependant in part on the number of cells actively proliferating in the tumor, it is important to know the actual proportion of multiplying cells. Frindel et al (1967) demonstrated that in an experimental fibrosarcoma there was a variation in the labeling index from one region of the tumor to another, although these regions did not differ in histological appearance. This signified that all cells of a particular tumor population may not be proliferating. The authors suggested that

such cells might be in the so-called G_o phase. Also Steel et al. (1966), using tritiated thymidine, demonstrated that there were varying numbers of proliferating cells in different tumor cell populations. They observed that 95 percent of a spontaneous mammary tumor was composed of a proliferating population, whereas a fibrosarcoma (BlCR/A2) contained merely 30 percent proliferating cells. Thus the growth characteristics of any tumor will depend on the proportion of proliferating cells in the total cell population. However, Baserga, Kisieleski and Halvorsen (1960) demonstrated in both solid and ascitic tumors that nearly 100 percent of the tumor cells can become labeled with tritiated thymidine. This observation would point to the conclusion that most tumor cells are viable and potentially proliferating.

Mendelsohn (1965) stated that, "if one accepts the concept of non-proliferating tumor cells, one is committed to the proposition that cell cycle time can not be estimated from growth curves, mitotic index determinations or techniques such as colchicine blockade and accumulation of mitotic figures as a function of time."

Just as cell production is important in the overall growth of a tumor, so is cell loss. Cell loss is not only a characteristic of tumors, but it differs from normal tissue in that there is usually no equilibrium between cell production and cell loss, as occurs in normal renewing cell populations. Cell production greatly exceeds cell loss in most tumors (Bertalanffy, 1967), thus resulting in a gradual cell increase or growth of the tumors. Cell loss is a difficult parameter to measure, but can be indicated, at least theoretically, by the discrepency between the total

cell production rate and the overall growth rate. Steel (1968) believed that cell loss is one of the major factors in producing a Gompertzian curve. Cell loss in general is ascribable to various inherent chracteristics of tumors: cell death, metastasis, and exfoliation. The causes of cell death may be brought about by a gradual increase in the host's response to the tumor, or a failure of the tumor to maintain a uniform blood supply because of its rapid growth rate. Yet, Laird (1964), believed that insufficient blood supply played little part in the retardation of tumor growth. Although metastases leave the primary tumor mass during various stages of tumor growth, they are not throught to contribute much to the gradual decrease in tumor growth with time.

Other factors responsible for a decline in tumor growth with time may be associated with the growth characteristics of the individual tumor cells themselves. Laird (1964) observed that the deceleration of tumor growth was caused, at least in part, by an actual increase in the generation time as tumor growth progressed. Lala and Patt (1966), working with ascites cells, likewise described a gradual prolongation of the phases of the mitotic cycle, together with a progressive decline in the growth fraction. However, studies on a fibrosarcoma indicated that the cell cycle did not change when progressing from one phase of tumor growth to another (Frindel et al., 1967).

It is thus apparent that the tumor growth rate depends chiefly on: 1) the cycle phase distribution of proliferating cells, 2) the proportion of nonproliferating cells (if any) in the tumor cell population and 3) the

extent of cell loss. However, the degree to which each of these factors influence the overall tumor growth varies from tumor to tumor, and the phase, early or later, of tumor growth studied.

As more and more tumor populations are investigated, with regard to their growth behaviour, it becomes evident that many tumor cells proliferate actually slower than the cells of some normally proliferating tissues. Thus, Bertalanffy and Lau (1962) demonstrated that the cells of some rat tumors proliferated slower than normal tissues, such as the pyloric mucosa (Bertalanffy, 1960).

CYTOSINE ARABINOSIDE

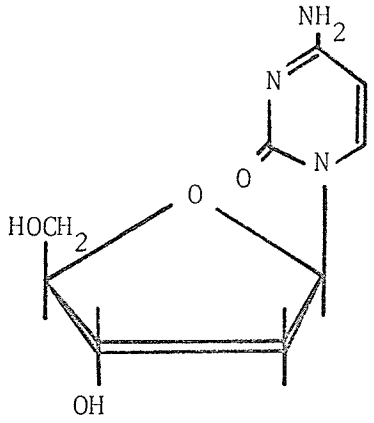
Introduction: Cytosine arabinoside (ara-C, cytarabine, CA, or 1 - β - D - arabinofuranosyl cytosine), is an abnormal pyrimidine nucleoside with interesting antitumor and antiviral activities.

Chemical structures and synthesis: Ara-C is an analogue of cytidine where the hydroxyl on the C2' of the sugar is in the cis position to the glycosyl linkage rather than trans as in the ribonucleosides. The sugar component is D-arabinose instead of D-ribose or D-deoxyribose, as occurring normally in ribonucleic and deoxyribonucleic acids respectively. The structures of the cytosine nucleosides are compared in Figure 2.

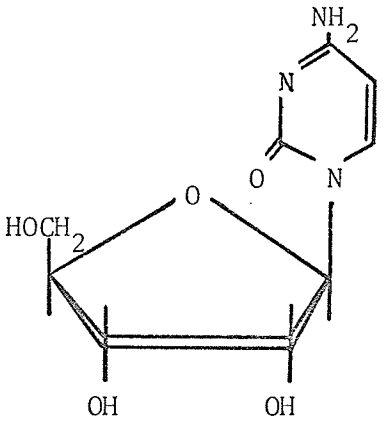
The arabinose-containing nucleosides of thymidine and uracil have been isolated from the sponge Cryptothelia crypta by Bergmann and Feeney (1951), and Bergmann and Burke (1955), respectively. Cytosine arabinoside on the other hand, has not been discovered so far as a naturally occurring compound, although it has been chemically synthesized, originally by Walwick et al., (1959).

FIGURE 2

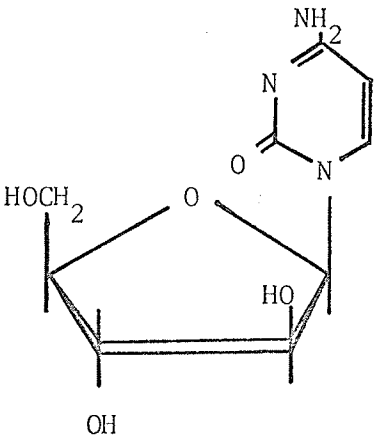
STRUCTURE OF CYTOSINE NUCLEOSIDES



Deoxycytidine



Cytidine



1-β-D-arabinofuranosyl

Cytosine

Effects of ara-C: Pizer and Cohen (1960), along with Slechta (1961), demonstrated that ara-C inhibited the anaerobic growth of Escherichia coli. Its anti-tumor activity was subsequently shown, first in a variety of mouse tumors by Evans et al. (1961, 1964b). These authors also demonstrated that ara-C had no significant activity against a spectrum of rat tumors or leukemias. Wodinsky and Kenster (1965) demonstrated a precipitous decrease of ascitic leukemic L1210 cells after ara-C (80 mg/kg) was injected intraperitoneally into mice on the fifth day after tumor transplantation.

Evans and Mengel (1964), working with two ascites systems (L5178Y and Ehrlich carcinoma) and one solid tumor system (T-4 lymphoma), showed that the antitumor effect of ara-C could be reversed by deoxycytidine (Cd-R). The importance of this reversal observed in vivo, of the inhibitory effect of ara-C by deoxycytidine explains the possible mechanism of ara-C action which will be discussed later. However, Chu and Fisher (1968a), using high concentrations of ara-C, observed that acute cell death in murine leukemia could not be reversed by Cd-R.

Evans et al. (1964a) also stated that porfiromycin and ara-C acted synergistically when administered conjointly, increasing the median survival time and the number of surviving animals. Kimball et al. (1966), working on the inhibitory effects of the arabinosides of 6-mercaptopurine (6-MP) and cytosine on Ehrlich ascites cells reported that these compounds increased the survival time of tumor-bearing mice. It was shown that 6-MP and ara-C had an additive effect upon the tumor growth in mice when the two were administered together. Tyrer et al.

(1967) studied the effects of ara-C and 1, 3-bis(2-chloroethyl)-1-nitrosourea on mouse leukemia L1210; he observed that a combination of the two chemicals was much more effective than either one alone. Hoffman et al (1968), working with advanced L1210 leukemia in mice, disclosed that sequential therapy of cytoxan and ara-C more than doubled the life-span of the animals over that of single treatment by either drug. They suggested that cytoxan "primes" the cells and makes them more responsive to ara-C treatment. Vadlamjdi et al (1968), similarly studying L1210 mouse leukemia, demonstrated that ara-C increased the survival time by 60 percent over the control animals. They observed further that a pre-treatment with colcemid, supposedly synchronizing the cell population, followed by ara-C treatment, further increased the survival time to 160 percent over the controls.

Harris and Hersh (1968) observed an immuno-suppressive effect of ara-C on the response of mice to sheep's red blood cells. This immuno-suppressive action of ara-C was thought to be a blocking of a division step in macrophage production, a requisite for the subsequent uptake and processing of antigen. Karnofsky and Lacon (1966) demonstrated that early treatment with ara-C on developing chick embryos caused greater abnormalities than if the treatment was administered after eight days of development.

Ara-C, apart from acting as an inhibitor of bacterial and tumor growth, as an immunosuppressor and as a teratogen, has other more direct effects upon the cell.

Both cytogenetic and morphologic changes have been shown to take place in cells under the influence of ara-C. Such effects caused by ara-C treatment were observed particularly in bone marrow cells (Talley and Vaitkevicius, 1963). Soon after treatment with ara-C the cells appeared puffy and distended taking on the appearance of cells in megaloblastosis. This morphological change is understandable as many workers have shown that ara-C specifically inhibited DNA synthesis while RNA and protein synthesis continued. The increase in cellular constituents has been compared to the enlarged appearance of mammalian cells under the effects of X-irradiation (Whitmore et al., 1958). Heneen and Nichols (1967) related this morphological appearance of cells treated by ara-C to the unbalanced rowth induced in a thymine-less state in bacteria, whereas Karon et al., (1966) have equated this cell enlargement to blastic transformation. These authors demonstrated further that the nuclear volume increased likewise after ara-C treatment. It was first believed that the DNA content increased but, this impression may have arisen because of a decreased compactness of nuclear material, indicated by a fainter staining reaction. Heneen and Nichols (1967) observed an augmented nucleolar activity as indicated by darker staining nucleoli after treatment.

Many authors reported cytogenetic defects after ara-C treatment (Kihlman et al., 1963; Block et al., 1965; and Brewen and Christie, 1967). Such effects appeared characterized by chromatid breaks, despiralization, stickiness and severe chromosomal fragmentation. These abnormalities were observed in non-dividing cells of the erythroid series,

but were absent in granulocytic precursors (Bell et al, 1966). Such aberrations are not believed to be caused by the inhibition of chromosomal DNA, but rather by abnormalities in the nucleoside pool formation (Brewen and Christie, 1967). This type of aberration induced by ara-C occurred even in unlabeled G_2 cells (Brewen, 1965). Brewen, (1965) and Brewen and Christie (1967) suggested that ara-C was not only a potent inhibitor of DNA synthesis but also a chromosomal breaker. These effects of ara-C do not seem to be causally related, inasmuch as chromosome breakage can occur during the G_2 phase of the cell cycle when DNA synthesis does not occur (Brewer and Christie, 1967). Recently, Brehaut and Fitzgerald (1968) supported this dual action view of ara-C by describing a high number of mitotic figures containing abnormal chromosomes merely three hours after ara-C treatment. These observations indicate that chromosomal breakage occurred during the G_2 phase of the cell cycle.

However, it has been found on cessation of low dosages of ara-C therapy that morphological and cytogenetic abnormalities returned to normal within varying periods of time (Block et al, 1965; and Bell et al, 1966). An explanation for this recovery from severe cytogenetic changes may reside in the structure of ara-C which inhibits DNA synthesis without becoming incorporated into the DNA molecule (Cardiellac and Cohen, 1964), and prolonged effects are thus possibly prevented. Other reasons may be ascribable to the rapid excretion of ara-C by most mammals, aiding in the rapid recovery of the cells. Yet, it was shown that high concentrations of ara-C may kill cells outright, and that a reversibility of its effects did not occur after removal of the drug (Heneen and Nichols, 1967).

Ara-C affects cells in two specific phases of the cell cycle. Firstly, it may break chromosomes in cells during the G_2-phase of the cycle, second, and more important, it halts DNA synthesis in cells during the S-phase of the cell cycle. The latter effect of ara-C has several implications on the cell cycle of both normal or abnormal cell populations.

Kim and Eidinoff (1965), working with cultured Hela S-3 cells, demonstrated an abrupt reduction of mitotic figures five hours after addition of ara-C to the culture media. There was a complete absence of mitotic figures as long as ara-C was maintained in the media. This observation was verified also by Brewen (1965) using human leukocytes. Ara-C either arrested all cells at the beginning of DNA synthesis or prevented cells in S from completing DNA synthesis. Thus, for a period of time, equal to or greater than the duration of G_2 plus M plus G_1, the inhibitory effect of ara-C produces an accumulation of cells at the beginning of the S-phase. This phenomenon was demonstrated by Kim and Eidinoff (1965) with Hela-S-3 cells, which when released from ara-C inhibition displayed a burst of mitotic activity. Ara-C can thus lead to a certain degree of synchronization of cells. Heneen and Nichols (1967) observed that the mitotic inhibition of WI-28 cells, a human diploid strain, by ara-C treatment was dependent upon the concentration and duration of the treatment. Low concentrations of ara-C allowed some post-treatment mitotic activity, whereas higher concentrations permitted none. Chu and Fisher (1968b) observed a slower generation time of treated L5178Y cells (of 23 hours) as compared to that of untreated (of 10 hours) L5178Y cells. This

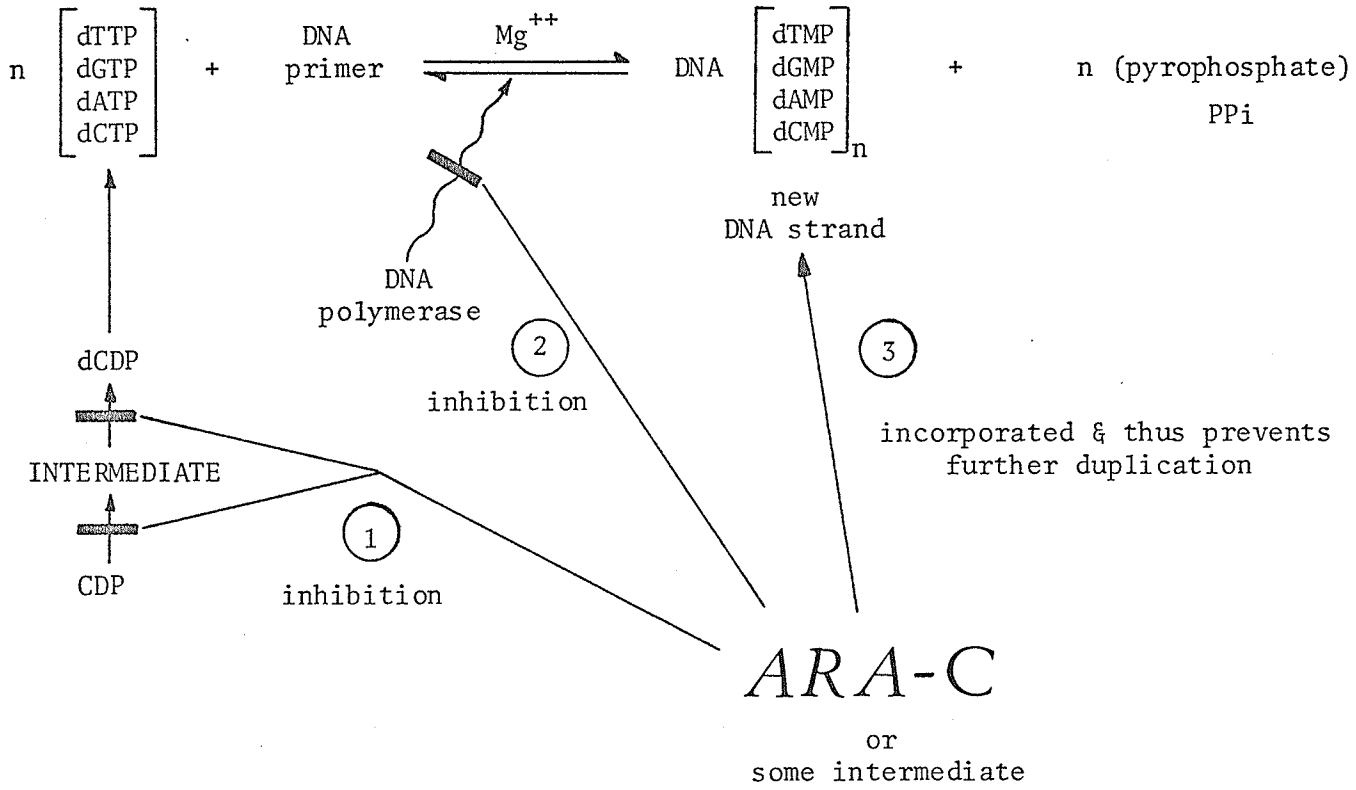
prolonged generation time was the result of cell death, since it had been shown that acute cell death (Brehant and Fitzgerald, 1968) but not delay of cell division was the principal toxic effect of high levels of ara-C.

Mechanism of action: The exact mechanism by which ara-C inhibits the synthesis of DNA and cellular reproduction, has not yet been fully elucidated.

To begin with, it was proposed by Chu and Fisher (1962), working with murine leukemic cells, and later by Cardeilhac and Cohen (1964), that the primary site of action of ara-C was an inhibition of the formation of deoxycytidine diphosphate (dCDP) from cytidine diphosphate (CDP). This inhibition prevented the synthesis of new DNA, and thus the cellular reproduction. Chu and Fisher (1962) likewise demonstrated that ara-C was a specific inhibitor of DNA synthesis, while RNA and protein synthesis remained unaffected. From studies of mutant strains resistant to ara-C, it was concluded that the enzyme phosphorylating cytosine arabinoside is deoxycytidine kinase (Chu and Fisher, 1965; and Momparler et al, 1968). Schrecker and Urshel (1968) concluded that the phosphorylation of ara-C by deoxycytidine kinase was a prerequisite for its antitumor activity. The reversal of the antitumor effect of ara-C by deoxycytidine (Cd-R), as shown by Evans and Mengel (1964), was explained by the hypothesis that a phosphorylated derivative of ara-C inhibited the reduction of CDP to dCDP, and that the formation of dCDP and deoxycytidine triphosphate (dCTP) from deoxycytidine could circumvent this inhibition (Chu and Fisher, 1962). Preventing the formation of dCDP by ara-C also caused a lowering of the

FIGURE 2a

POSSIBLE SITES OF ARA-C ACTION ON THE OVERALL PROCESS OF DNA SYNTHESIS



dCMP pool and a decreased thymidylate synthesis, causing a stimulation of uptake of exogenous thymidine by means of thymidine kinase. This action would explain the continued uptake of 3H-thymidine by cells which remain arrested in the S-period by ara-C, primarily because of the depressed dCTP levels (Brehant and Fitzgerald, 1968). Inasmuch as ara-C became phosphylated to dCTP on its uptake into cells, it was proposed that it was incorporated into RNA and DNA fractions in internucleotide linkages (Chu and Fisher, 1965). Such an incorporation of ara-C into the DNA and RNA of mouse fibroblasts (Silgi, 1965), and human leukemic cells (Creasey et al., 1966) was reported. It stands to reason that such an incorporation of an ara-C analogue into nucleic acids might account for its toxic effect on cells. More recently, Chu and Fisher (1968a) showed in a detailed report that 3H-cytosine arabinoside was incorporated into both RNA and DNA fractions. Regarding the metabolic lesion responsible for ara-C - induced acute cell death, this has not been fully elucidated.

Metabolism of Ara-C: Ara-C was shown to become rapidly deaminated to uracil in bacteria (Pizer and Cohen, 1960, and Slechta, 1961). Deamination of ara-C into inactive ara-uracil in mice (Camiener and Smith, 1965; and Saslow et al., 1966), in cultures of mammalian cells (Smith et al., 1965; and Dollinger et al., 1967) and in human patients (Creasey et al., 1966) was reported. Camiener and Smith (1965) observed that in mice, ara-C became deaminated in the liver. They concluded that most of the ara-C was removed from the blood by the liver, but that other pathways for detoxifying ara-C were also possible. It is interesting to note that in rats, an effect of ara-C on cells and cytidine deaminase was not observed

in a number of tissue studies (Camiener and Smith, 1965; and Wodinsky and Kenester, 1965). Skipper et al. (1967) determined the blood levels of ara-C at various intervals after a single intraperitoneal (i.p.) injection and after a constant i.p. infusion of mice. The in vivo half-life in the blood of mice was of the order of 10-20 minutes, with maximum levels attained at about 15 minutes after i.p. injection, and at about two minutes after intravenous (i.v.) administration. This observation was recently confirmed by Borsa et al. (1969) who demonstrated a half-life of 21 ± 8.4 minutes. They cited a paper by Mulligan and Mullet (1968) who found a 20 minute half-life after a 250 mg/kg dose, and a 37 minute half-life after a 50 mg/kg dose in BDF_1 mice. They could not explain, however, the increased half-life with the smaller dosage. Skipper et al. (1967) observed that single i.p. doses of ara-C in the order of 50 mg/kg did not result in effective blood levels for more than one to two hours.

Evans et al. (1964b) demonstrated that ara-C was about three times more effective when given parenterally than when administered orally. Kline et al. (1968), dealing with advanced leukemia in mice, observed that four daily oral applications of the drug did not increase the antitumor activity of ara-C but instead enhanced its toxic effects. Its poor antitumor activity was attributed to its deficient absorption (Dixon and Adamson, 1965) and its rapid deamination to ara-U, an inactive metabolite.

Kessel (1967) showed that the rate of ara-C nucleotide formation increased with elevated ara-C levels, thus increasing its effectiveness. Ara-C does not diffuse freely into cells, but uptake of the drug by the

cells is mediated by its intra- and extracellular concentrations (Kessel et al, 1967).

The chemotherapeutic action of a drug was shown not to be a fixed property of the drug, but rather dependent on and influenced by alterations in the schedules of treatment (Kline et al, 1966). Evans et al (1964b), studying the antitumor activity of ara-C on experimental murine tumors, reported that a high single parenteral dose of ara-C, 24 hours after tumor transplantation, yielded an increased survival time of the animals. However, they observed that the response was greater when the drug was administered at a lower total dosage level over a longer period of time.

Skipper (1965) postulated that any efficient chemotherapeutic drug dose must kill every tumor cell without killing the host, regardless of the total number of tumor cells present, their anatomical position, or metabolic heterogeneity. He also argued that the drug should kill sufficient numbers of tumor cells so that those remaining could be killed by host mechanisms. Skipper et al (1965) reported that the percentage of kill, or fractional reduction of cell populations of various sizes was reasonably constant for a given dosage of an antitumor agent.

Kline et al (1966, 1968) likewise demonstrated that two or four daily subcutaneous injections augmented the antileukemic effect over a single daily injection. In essence, the blood level of ara-C was kept more constant with multiple injections than with a single higher dose. This chemotherapeutic approach is reasonable, inasmuch as it was shown by Dollinger et al (1967) and others that ara-C becomes quickly deaminated

to uracil. Skipper et al. (1967) observed that eight injections over a 24-hour period kept the ara-C blood levels sufficiently high to affect the leukemic cells. They allowed a three day period following each 24-hour injection period, for host recovery. Skipper et al. (1967) felt that such an attack on the S-phase of the cells would reduce the number of tumor cells sufficiently so as to implement a complete cure. However, they assumed either that cells were not hibernating in a so-called G_o phase, or were truly drug resistant. Vallamudi et al. (1968) concluded that a maximum number of L1210 leukemic cells could be affected by ara-C after synchronization of the cells by colcemid. It was shown by Kim and Eidinoff (1965) that ara-C may be used to obtain a certain degree of synchronization as cells already in the S-phase at the time of ara-C injection are halted in the S-phase, while those cells in G_2, M or G_1 are prevented later on from entering the S-phase. Wilkoff et al. (1967) observed that drug exposure, for a period, less than one-sixth of the generation time of the cell population was ineffective, regardless of the size of the dose.

It must thus be concluded that only a fraction of tumor cells, those in the S-phase, will be affected by ara-C at any one time. However, if the ara-C levels in the body fluids can be maintained fairly constant for a period equalling the generation time, a high percentage of cells would be killed. Yet, a period permitting the host to recover seems to be an important factor before complete irradication of the tumor population can be accomplished by subsequent treatment (Leach et al., 1969).

Clinical application of ara-C against leukemia and other human neoplastic diseases have so far met with merely modest success. The success achieved with ara-C depends both upon the dosage and the scheduling of administration (Burke et al., 1968). A patient with acute myelogenous leukemia was treated by a constant infusion of ara-C with merely a slight improvement (Carey and Ellison, 1965). Leukopenia rapidly occurred, while other toxic effects were confined to a mild nausea, stomatitis and phlebitis at the drug infiltration sites. These effects, in particular nausea and vomiting, were also observed by Bodey et al. (1968), and in ara-C treatment of acute childhood leukemia by Howard et al. (1968). Bodey et al. (1968) demonstrated that ara-C activity against leukemia could be improved by changes in scheduling, and when combined with cycle-sensitive agents such as cytoxan. Minimal tumor regression was observed without significant clinical benefit in a case afflicted with malignant melanoma (Papac, 1968).

An excellent clinical review of cytosine arabinoside was provided by Livingston and Carter (1968).

MELANOMA B16

Microscopic Structures. On gross examination, melanoma B16 tumor appeared as a soft, dense, black tissue, usually forming an encapsulated nodule (Green, 1968). However the cells of the melanoma occasionally invaded the surrounding tissues.

Microscopically, the individual tumor cells varied from round or polyhedral to spindle shaped depending on the area of the tumor examined.

The cells were arranged in diffuse masses or in bands about the numerous blood vessels. The size of individual tumor cells ranged from 12 - 16 μ in diameter, while the nuclear size and shape varied greatly. Nucleoli ranged from one to three per nucleus and the nucleus contained distinct chromatin material. The cytoplasm appeared moderately basophilic with hematoxylin and eosin, and sometimes contained melanin granules (Bertalanffy and McAskill, 1964). The melanin granules, if present, ranged in color from yellow to black or deep brown. The very word melanin comes from the Greek word melas meaning black (Attie and Khafif, 1964). Bertalanffy and McAskill (1964) noted that metastases, from primary melanoma tumors, in both the liver and spleen likewise exhibited the same general microscopic appearance as the primary tumor.

Wellings and Siegel (1960) observed with the electron microscope that the melanin granules of a human melanoma were 300 mμ in diameter, and consisted of granular and rod-shaped internal units. The melanin granules were shown to originate in the Golgi apparatus which was surrounded by a moderate abundance of mitochondria and ergastoplasm.

Growth. Various authors working with hamster melanomas found that only rarely did a transferred tumor fail to take. Kokame et al. (1960) demonstrated that hamster melanoma was not only readily transplantable to many sites in the hamster but that heterologous transplantation was possible in the subcutaneous space of Swiss mice, peritoneal cavity of fetal guinea pigs, and also the anterior chamber of the eye in adult guinea pigs. Rosenberg et al. (1961a), (1961b), Moore (1964),

and Greene and Harvey (1966), observed that heavily pigmented melanomas generally grew slower than amelanotic melanomas. Rosenberg et al (1961a) found the survival times in hamsters with heavily pigmented melanotic tumors to be in the range of 32-118 days, while the times with amelanotic tumors were only 22 - 88 days. The percentage of metastases in hamsters dying was higher in those hosts bearing amelanotic tumors than in others bearing melanotic tumors. Gray and Pierce (1964) demonstrated that single melanoma cells from hamster melanomas developed along six clonal lines. Two of these were heavily pigmented and slow growing, while the other four were lightly pigmented and grew rapidly. Amelanotic tumor cells were observed to grow faster than melanotic tumor cells also in their ascitic form (Gray and Pierce, 1964). The latter authors considered amelanotic tumor cells to be undifferentiated. Their data supported the concept that the growth rate and differentiation, that is melanogenesis, were inversely related in this melanoma of the golden hamster. This supposition was supported by Hu (1966), working with B16 mouse melanoma, who demonstrated that colchicine inhibited mitotic activity of the cells, was followed subsequently by an enhanced pigment cell formation.

The only reference to the actual growth rate of malignant melanoma was provided for the B16 malignant mouse melanoma by Bertalanffy and McAskill, 1964. This tumor was observed to grow at a constant rate in C57BL/63 mice by a daily addition of 34 to 36 percent of new cells to its population, implying a tumor doubling time of 2.8 days. The growth rate

of metastases in the liver and spleen was similar to that of the transplanted primary tumors. Bertalanffy and McAskill did not observe significant diurnal fluctuations of mitotic activity in the B16 melanoma. Helpap and Maurer (1967) reported in an abstract that the duration of the S-phase of the B16 melanoma cells was seven hours both in vitro and in vivo.

Effect of Hormones on Melanoma Growth. Hormones have shown wide and varying effect upon melanomas of both human and animal species. In man, statistical analyses indicated that hormonal factors influenced the growth of melanomas significantly, with the male hormonal milieu favoring the growth rate, whereas the female milieu failed to affect, or possibly even depressed growth of the tumors (White, 1959).

In animal investigations, hamster and mouse melanomas were studied most widely. In consideration of the above, it seems surprising that in hamsters the female hormonal milieu was more conducive to tumor growth than the male hormonal milieu, that is, the complete reversal of humans (Rosenberg et al, 1963).

DasGupta and Terz (1967a, 1967b) reported that melanoma tumors in pinealectomized hamsters attained much larger sizes than either control or sham-operated animals. They surmised that the pineal must have some control over the growth and spread of pigmented neoplasms. However, they were unable to demonstrate whether such control was via the effect of a pineal hormone on the melanophores, or an ablation of the pineal depressed host resistance to tumors and homografts. Hu (1966) did not observe any

effect on the amount of melanin granules in B16 melanoma cells of mice with melanin stimulating hormone (MSH) treatment. Thus, tumor growth could not be attributed in this instance to MSH. Retik et al (1962) showed that pregnancy in DBA mice enhanced the growth of transplanted S91 Cloudman melanoma tumors. They also observed that the growth of pulmonary metastases was inhibited, although their formation was not prevented. They suggested that the inhibition was possibly brought about by concurrent growth of the fetuses, which indirectly competed with the tumors for the nutrients. They excluded the possibility that the growth of the primary tumor affected that of the lung metastases, because an inhibitory effect was not seen in mated or non-pregnant control animals bearing primary tumors. Crile (1963) demonstrated that repeated injections of serotonin depressed the growth rate of S-91 melanomas on the feet of DBA/1 mice. Moreover, he observed that an injection of serotonin 30-40 minutes prior to a heat treatment of the feet, of 44^{o}C greatly enhanced the destroying power of the heat.

Effects of Drugs on Melanoma Growth. Among the possible approaches to provide rational chemical therapy for malignancies is one that centers about attacking a specific or exclusive aspect of the malignant cells' vital processes.

Fitzpatrick and Kukita (1959) demonstrated that pigmented malignant melanomas were characterized by containing tyrosinase in a highly active form, whereas in the normal resting adult mammalian melanocytes tyrosinase occurred in an inactive form. They demonstrated further that tyrosinase

activity directly paralleled the mitotic rate of melanocytes. In normal tissues both the tyrosinase and mitotic rate were high in retinal melanocytes during ocular development as compared to that during adult life. Likewise tyrosinase and mitotic activity were higher in melanomas than in pigmented nevi.

With this basic knowledge many authors employed various drugs known to inhibit tyrosinase activity in an attempt to combat malignant melanoma tumors. Tyrosinase is an essential catalyst in the first two steps of the conversion of tyrosine to melanin. Demopoulos and Kaley (1963) and Demopoulos et al (1965) suggested that tyrosinase was both a vital respiratory and a biosynthetic enzyme.

Luck (1961) established that the meta- mustard derivatives of phenyl-DL-α-alanine (PIA) was a marked inhibitor of S 91 Cloudman melanoma in male DBA/1 mice. PIA has been shown to inhibit oxygen consumption and explant growth of pigmented malignant melanoma both in vivo and in vitro (Demopoulos and Kaley, 1962, 1963; Demopoulos et al., 1965; Demopoulos, 1962, and Mitamura et al., 1966). However, the selective inhibitory effects of PIA required a transient period of anoxia or chilling for their induction in vivo against established tumors (48 or more hours after subcutaneous transplantation). Duke and Demopoulos (1967) reported that even large repeated injections of PIA failed to affect the growth of established S 91 melanomas. They also demonstrated that PIA and L-cysteine caused necrosis of large established S 91 mouse melanomas in vivo. Duke et al., (1967a) supported this finding, and also established that normal tissues

suffered no toxic effects; they thus assumed that PLA was specific to pigmented melanomata.

The B 16 mouse melanoma, which is a very rapidly growing, heavily pigmented tumor was shown by Demopoulos et al. (1965) to be resistant to phenyl lactate alone, but when treated very briefly with chilling or transient anaerobiosis, became very susceptable to it (Demopoulos et al., 1965). These authors also demonstrated that amelanotic human and non-pigmented S 91 melanomas did not have a segment of their respiration cycle sensitive to PLA.

Friedell et al. (1961) reported that methotrexate appreciably inhibited the growth of a human malignant melanoma ten days after administration. Hoye and Weiss (1966) demonstrated that laser energy combined with chemotherapy with cyclophosphamide was more effective as an inhibitor against S 91 melanoma in mice than either laser energy or cyclophosphamide alone. Penicillamine was shown to inhibit S 91 mouse melanoma in vivo but not in vitro (Duke et al., 1967b). Penicillamine treatment reduced the tyrosinace activity in the melanomas by reducing the amount of copper present, this being a cofactor for the enzyme. However, penicillamine did not produce a selective growth inhibition against melanomas, although it displayed some effects against cultured heart tissue. Heparin was used by Boeryd et al., (1967) to inhibit intravenously introduced B 16 melanoma metastases. They reasoned that the anticoagulant effect of heparin led to reduced thrombi formation and/or less retention of tumor cells in the lungs. Moreover, Boeryd et al. did not observe an increase in metastases

to the liver or other extrapulmonary sites. In the same study, both ϵ-aminocaproic acid and guanethidine administration resulted in an increase in number and total volume of metastases in the lungs.

Effects of other agents on Melanoma growth. Decosse and Rogers (1966) demonstrated that hamsters with AMEL-4 hamster melanomas, which normally killed the host between 7 - 10 days, showed a minor but significant improvement in survival when treated with high-pressure oxygen. They tested the effects also of mechlorethamine, cyclophosphamide and amethopterin on the tumor and observed a slight increase in survival times with the former drug and a moderate increase with the latter two chemicals. Synergism between any of these drugs and high-pressure oxygen was not observed.

Salo and Blair (1960), working with the Harding-Passey melanoma in the Rockland albino mouse reported that I^{131} injected in a minimum total dosage of 300 μC at the time of transplantation decreased the percentage of tumor takes. However I^{131}, even at higher dosages (450 μC), did not affect the growth rate once the tumor was transplanted. They also found that 400 μC injected directly into the rapidly growing melanoma did not inhibit the growth rate of the tumor.

Hunter (1955) observed that temperatures of 35^{o}C, although without detrimental effects on the animals, caused a moderate growth inhibition of the S 91A amelanotic melanoma. When the temperature was decreased to 25^{o} C, the inhibition was completely reversed in the S 91 tumor. Hunter attributed these observations partly to a differential response of these tumors to conceivable endocrine changes brought about by temperature

stresses upon the host. More recently, Crile (1963) found that by exposing to heat of 44°C for 30 - 40 minutes, S 91 melanomas transplanted to the feet of mice destroyed a high proportion of the tumor without any damage to the feet. Crile demonstrated further that moderate dosages of heat and irradiation, regardless of the mode of administration were synergistic or at least additive in their effects on tumor growth. It is interesting to note that after exposure to one-third of a lethal dose of heat both tumors and normal tissues became heat-resistant and were not damaged by subsequent exposures that would normally destroy them.

Minton and Ketcham (1964), applying laser radiation at 6,943 Å, completely destroyed the S 91 melanoma in the CDBA/$2F_1$ hybrid mouse. They concluded that permanent tumor destruction was dependent upon the amount of laser energy applied, and the size of the tumor implant to be destroyed. Hoye and Weiss (1966), working with the same tumor but in the CDF_1 mouse, discovered that laser energy along with cyclophosphamide, was more effective in irradicating the tumor than either the drug or laser alone.

It is evident from the search of the literature that much more work has to be undertaken to augment our understanding of the vulnerability of the malignant melanoma.

EHRLICH ASCITES TUMOR

Introduction: Ehrlich ascites tumor cells are high grade malignancies which were derived from a spontaneous mammary gland adenocarcinoma in 1905. However, since that time numerous strains of Ehrlich ascites tumor cells

have evolved and perpetuated. The free ascitic cells are propagated by injecting them, suspended in ascitic fluid, into the peritoneal cavities of mice. As the cells proliferate they exist in and elaborate an increasing amount of exudate. It was assumed by Lettre (1941), and confirmed by Klein and Revesz (1953), that the increase of ascitic fluid was directly proportional to the increase in the number of tumor cells during growth. Following their division, the newly formed tumor cells occur free in the exudate, and exist as physically independent units (Lucke and Berwick, 1954). The survival time for mice bearing such tumors is about two to three weeks at the most, depending on the dose of tumor cells.

Microscopic Appearance: Microscopically, Ehrlich ascites tumor cells range considerably in size. They vary from small cells with moderately sized nuclei to larger cells with coarsely granulated chromatic nuclei. Many of the nuclei are hyperchromatic because of excessive amounts of DNA present (Bertalanffy et al., 1965).

Agnish and Fedoroff (1968) studied two, near tetraploid, strains of Ehrlich ascites tumors. Determining the cell volume, staining characteristics, chromosome number, and DNA content measurements, they distinguished between three types of cells. One type was represented by small cells with purple-staining nuclei, another by medium sized cells, and the third by vacuolated cells. The latter two types were thought to be the same cell category but in a different functional state. Host lymphocytes were also found among the tumor cells.

Growth of Ehrlich Ascites Tumor (EAT) Cells: The growth patterns of Ehrlich ascites tumors have been studied for almost forty years. Early

workers in this field were Klein (1950); Goldie and Felix (1951); and Yoshida (1952).

Ehrlich ascites tumor (EAT) cells belong to the fastest growing cell populations, and approach the growth rate of mouse embryo tissues; but they do not exceed it (Klein and Revesz, 1953). The growth pattern of the EAT cells was first demonstrated by Klein and Revesz (1953), and subsequently by Patt and Blackford (1954), to comprise two distinct phases. The first phase extended from the time of innoculation to approximately the end of the seventh day, and is the so-called exponential phase. During this phase the number of tumor cells in the peritoneal cavity increased exponentially. The late phase spanned the period from about one week after innoculation until death of the host. During this latter phase, the number of tumor cells in the peritoneal cavity increased only slightly, if at all. Lately, this two-stage pattern of growth was confirmed by the use of radioactive thymidine (Baserga, 1963).

It is important to realize that the slopes of the growth curves of mouse ascites tumors are variable, depending significantly on the strain of cell, the host, the size of the inoculum, and on other factors. It was established by Klein and Revesz (1953), that a latency period followed the innoculation of ascites cells into a host animal. During this period, the cell number remained stationary or even decreased slightly. This latency period varied inversely with the size of the innoculated dose. With an innoculum dose of 0.7×10^6 cells or more, the period became immeasurably short (Klein and Revesz, 1953). In an innoculum from an old host containing sufficient cells so as not to produce an appreciable lag,

there was a rapid rise in the growth fraction of the tumor cells when they were re-injected into a new host. This indicated that most of the tumor cells retained a potential proliferative capability, although they may have been in a resting stage (G_o). During this period, immediately after transplantation, the percentage of cells in the population synthesizing DNA or undergoing mitosis was increased, as evident from their augmented mitotic index. Lala and Patt (1968) postulated that this augmentation of the mitotic index could only be explained by a prompt shortening of other stages of the cell cycle in relation to mitosis. Both Lala and Patt (1966), and Wiebel and Baserga (1968), established that the cell cycle times were significantly shortened after transplantation. Wiebel and Baserga (1968) suggest that some stimulus triggers these cells into an immediate DNA synthesis once they have been transplanted. In Figure 3, the duration of the cell cycle times and the individual phases of the cycle of various strains of Ehrlich ascites tumor cells are compiled. It is interesting to note that Baserga (1963), working with a hypotetraploid subline of Ehrlich ascites tumor cells, could not detect any G_1 phase of the cell cycle. Thus these tumor cells, immediately upon division, commenced to synthesize DNA for a subsequent division.

Baserga (1963) stated that since approximately one half the cell population, during the exponential growth, phase of the tumor, became labeled, they were acting as a veritable trap for nucleic acid precusors.

Bertalanffy et al. (1965), in an interesting paper, compared the doubling time of EAT cells grown both in ascitic and solid forms, using

FIGURE 3

CELL CYCLE TIME (HRS) OF VARIOUS STRAINS OF EHRLICH ASCITES TUMOR CELLS ON DIFFERENT DAYS AFTER TRANSPLANTATION

t_C	t_S	t_{G2+M}	t_{G1}	DAYS OF TUMOR GROWTH	STRAIN	REFERENCE
18	12	6	0	-	tetraploid	Hornsey & Howard,1956
18	8.5	1.5+5.1	3	*	-	Edwards et al, 1960
18	11	6.5	0.5	¼	hypo-tetraploid	Baserga & Gold, 1963
18	11	6	0	4	"	Baserga, 1963
18	11	6	0	7	"	"
18	11.5	6.5	0	13	"	"
19	8.5	3.8+1	5.7	4-5	-	Kim & Evans, 1964
36	19	5.5+0.5	11	6	-	Baserga et al, 1965
8	6	2+t_{G1}	0	1	hyper-tetraploid	Lala & Patt, 1966
17	13	4	0	4	"	"
22	18	4	0	7	"	"
47	26.5	5.5+1	14	5	hypo-tetraploid	Wiebel & Baserga,1968
21.5	16	4+1	0.5	¼	"	"
8	6	1.2+0.8	0	1	hyper-tetraploid	Lala & Patt, 1968
22	18	2.2+1.8	0	7	"	"
15	8	3-4	2-3+4	6-8	hyper-diploid	Lozzio, 1968
8-47	6-26.5	2.0-6.6	0-14		RANGE (HRS)	

* during Log. growth.

the colchicine method. The doubling time of the ascitic form of EAT cells was determined to be 21 hours, while the doubling time of the same cells, grown in the subcutaneous fascia, became extended to 29 hours. These observations, and in particular the doubling time of the EAT cells, agreed with those of other authors, although the methods and strains of tumors used varied. These authors observed further that diurnal variations of mitotic activity did not occur in the ascites tumor.

During the later or terminal phase of ascitic tumor development, there is almost a complete lack of growth. Klein and Revesz (1953), plotted the cube root of the total number of tumor cells in the peritoneal cavity, demonstrated a straight line for the exponential growing period with time. After about six days the cube root of the tumor cell number no longer conformed to this picture of exponential growth but began to decline. Twelve days after tumor transplantation, this growth curve neither decreased or increased but remained stationary. Assuming that cell death was negligible, this would seem to signify a complete lack of growth. The small amount of cell loss displayed throughout the phases of growth would probably prove ineffective.

It was concluded from these observations that the gradual retardation of the rate of Ehrlich ascites tumor growth with increased tumor mass was brought about by a gradual prolongation of the phases of the cell cycle, and a progressive decline in the growth fraction. Lala and Patt (1966) established a cell cycle time of only 8 hours during the early exponential growth phase of a hyperdiploid Ehrlich ascites tumor. They found that in the later stages of growth, the phases of the cell cycle were prolonged.

Surprisingly, the prolongation mostly affected the S and G_2 phase of the cycle. Prior to that observation, the S-phase was considered to be the only constant phase of the cycle. These authors were unable to demonstrate an existing G_1 phase during the exponential growth phase, nor did one appear when the cell cycle became prolonged during the later stages of growth. It is interesting to note that cells from this latter phase of growth, when injected into a new host, exhibit after a variable lag period a shortening of their cell cycle duration (Wiebel and Baserga, 1968). Presumably the durations of G_2 and S phases become briefer to accommodate the decreased cell cycle. The rapid rise observed in the number of cells labeled with tritiated thymidine suggested not a shortening of the cell cycle but rather an entry into S from either a suspended S or G_1 phase (Lala and Patt, 1968).

Effect of Sex on Growth: It was known for some time (Bittner, 1932) that the sex of the recipient animals influenced the growth of some transplantable tumors. For some reason, females were more favourable to tumor growth than males, possibly because the hormonal milieu of the female was more conducive to the small portion of transplanted tumor cells. Most Ehrlich ascites tumor cells injected into the tail vein of mice usually failed to survive at the site of arrest in the lungs. However, a greater number of EAT cells survived in the female than in the male, as reported by Baserga and Kisieleski (1960). These authors were quick to point out however, that those cells surviving, in both male and female, grew at a similar and constant rate. However, in Strong A mice, the doubling time

of the EAT cells growing in the peritoneal cavity was twice as long in the females (36 hours) as in the males (18 hours) (Baserga, 1963). The cell cycle times were T_C= 18 and 24 hours, T_s = 10.4 and 12.5 hours, T_{G_2} = 6 and 6 hours and T_{G_1} = 1.6 and 5.5 hours in males and females, respectively, in Strong A mice (Baserga and Lisco, 1963). Consequently, the tumor grew faster in the males than females. However, more recently in a study by Vincent and Nicholls (1967), investigation of the effects of sex on EAT cell growth indicated a slower growth rate in males. They demonstrated that significantly less growth occurred with the tumor samples from male donors when tested in both males and in females, than those from female donors. Moreover, slower tumor growth was observed in tumors transferred into males from either male or female donors. These observations suggested that in this experimental situation the slower growth of the male animals over female animals may have been attributable to a selection of cells with a decreased growth potential in males. However, it should be kept in mind that the reverse has been shown as well, in regard to the effect of sex upon growth.

Effect of Drugs on EAT Growth: EAT cells have been widely employed as a tumor model on which to test many antimitotic drugs. The prime reason for the selection of EAT cells as a model is their brief doubling time, and secondly, the ease with which tumor cells can be transferred and harvested.

Many drugs have been tested on EAT cells to ascertain their effects upon the life of the tumor cells. As the numbers of drugs thus employed are too numerous to discuss, merely a few of the more recent ones will

be examined.

With most of the drugs and methods used to combat the growth of tumor cells, a specific segment of the cell cycle is usually the target for the action of the drug or treatment. Nitrogen mustard* was tested on EAT cells and was observed to block the life cycle at the G_2 phase (Layde and Baserga, 1964). The uptake of DNA precursors were slowed while protein and RNA precursors were unaffected. The prime effect of nitrogen-mustard is a premitotic block, while the decreased uptake of DNA precursors are secondary. It is interesting to note that within 24 hours after the exposure of the G_2 cells to nitrogen mustard, such cells by pass mitosis (the division stage) and commence again to duplicate their DNA, producing a cell with a double amount of DNA. In this manner, nitrogen mustard simply delayed the cell cycle and thus decreased the generation time. Another compound used to combat the growth of tumor cells is vincaleucoblastine (VLB). This compound, a metaphase inhibitor at doses similar to colchicine, extended the generation time of EAT cells by 6 times, as reported by Schachter,(1965). This author discovered that two doses were more effective than a single application, and that the number of doses was more decisive for the effect of the drug than the intervals between injections.

In EAT cells grown in Strong A mice (Baserga et al., 1965) it was the G_1 phase that was sensitive to small amounts of actinomycin D(0.016 µg/gm body weight). Actinomycin D*, in the proper dosage, arrested G_1 cells, whereas it had no direct effect on S, G_2 or M phases. With the

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* See addendum, p. 127a

continuous flow of the cell cycle, this resulted in a gradual decrease in the number of cells entering the S phase. Actinomycin D inhibited DNA-dependent ribosomal RNA, which became reduced by 50 percent, within 30 minutes after the first injection. DNA synthesis was not affected for 270 minutes after the first injection, and became fully inhibited only after 510 minutes. Baserga et al., (1965) reported the block to occur precisely 2 hours before onset of S; thus the late G_1 phase is the vital period of time.

In an in vitro study Basrur et al. (1968) observed that ethylheptyloxyacetate within four hours depressed the percentage of labeled cells to approximately two-thirds over the control EAT cells. This marked decrease in labeled cells, undergoing DNA synthesis at the time tritiated thymidine was available, was brought about by a destruction of cells during mitosis at the time of treatment. There was a complete recovery of the percentage labeled cells 24-48 hours after treatment, but the complete lack of mitotic figures persisted until 48 hours. The recovery of the labeling index within 48 hours was explained by the authors by a delay of the cells in G_2, which then bypassed the mitosis phase and began to re-duplicate DNA, similar to cells treated with nitrogen mustard (Layde and Baserga, 1964). Kito (1968) demonstreated that oxygen high pressure (OHP) ((4 atm. abs.) decreased the total number of a hypotetraploid strain of EAT cells and thus prolonged the survival time of the animals. However, he also demonstrated that OHP was more effective when the treatment was combined with methyl-bis (2 - chloroethyl) amine N - oxide

hydrochloride (HN_2 - O). Other agents proved ineffective on the growth of EAT cells. For instance, Hanson and Bohley (1968) were unable to demonstrate any deleterious effects on EAT cells of anionic, cationic and nonionic detergents alone or in combination with degranol or D2-glyceraldehyde.

A great deal of attention has been directed to the effects of irradiation on the cell cycle of many tumors. Kim and Evans (1964), working on six day old EAT cells, demonstrated certain phases of the cell cycle to be more radiosensitive than others. The G_2 and S phases appeared more sensitive to irradiation than either the M or G_1 phases. EAT cells irradiated while in G_2 exhibited the greatest mitotic delay. With a 500 rad. dosage of x-irradiation, the duration of S became extended from a normal 8.8 hour duration to 15 hours. Such effects of x-irradiation were shown with a similar dosage also for ELD ascites cells by Lozzio (1968). Plotting a curve of labeled mitoses as a function of time indicated an apparent shortening of the S phase. Lozzio suggested that this shortening could be related to an unequal delay of the cells irradiated during S, for cells irradiated later during the S phase became more delayed than those irradiated earlier during the phase. He concluded thus, that the actual effect of x-irradiation on the S phase may be an extension, as shown by Kim and Evans (1964).

A compound related to cytosine arabinoside, 9-D-arabinosylpurine, was studied as to its effects on the growth of ascites tumor cells. Brink and Lepage (1964) observed that adenine arabinoside (ara-A) inhibited growth

of TA3 and 6C3HED ascites tumor cells. Yet, ara-A exerted little effect on the ascites, strain L1210. Using a dosage of 25mg/kg twice daily for six days they found that the survival time became extended to 50 days, and the mice became apparently tumor free. In another study using ara-A, York and LePage (1966) observed that the mechanism of action of the drug was brought about by a non-competitive inhibition of DNA polymerase in TA3 cells. It is important to emphasize that cytosine arabinoside, a pyrimidine analogue, may act in a different manner than ara-A. Ara-C's primary site of action was a prevention of the formation of deoxycytidine diphosphate from cytidine diphosphate (Chu and Fisher, 1962; and Cardeilhac and Cohen, 1964).

Yet, Kimball and Wilson (1968), studying the effects of ara-C on EAT cells in female Swiss mice, established that the mechanism of action was an apparent inhibition of DNA polymerase, similar to that observed for ara-A. In EAT cells, ara-C inhibited both <u>in vivo</u> and <u>in vitro</u> utilization of thymidine for DNA synthesis. The authors reported also that this inhibition could be reversed by the action of deoxycytidine-5-triphosphate.

CHAPTER III.

MATERIALS AND METHODS

ANIMALS USED

In the melanoma experiments, male C57BL/6J mice (JAX) (Roscoe B. Jackson Memorial Laboratory; Bar Harbor, Maine) 5 - 6 weeks old and weighing 18 - 26 gms. were used.

For the ascites experiments, male albino mice (Connaught Laboratories; Willowdale, Ontario) 5 - 6 weeks old and weighing 15 - 20 gms. were used.

All mice were housed four per cage and given Purina Fox Chow and tap water ad libitum. The animal room was maintained on a diurnal lighting schedule (daylight: 8:00 a.m. to 8:00 p.m.) and kept at 78± 2°F.

TUMORS USED

Melanoma tumor The B16 melanoma tumor was obtained from the Jackson Laboratory (Bar Harbor, Maine). It arose spontaneously in the skin, at the base of the ear, of a C57BL/6J mouse in 1954 (Green, 1968). As of December 1967, this tumor had undergone 328 transfer operations since 1954.

Ehrlich ascites tumor A type of Ehrlich-Lettre ascites tumor was used and has been chromosomally identified to be hypotetraploid, with cells containing predominately 71 chromosomes, one of which was a biarmed marker chromosome. The tumor strain has been maintained since 1964 by Dr. C. M. Dowse, Faculty of Dentistry, University of Manitoba.

Chromosomal Analysis

The basic procedure used for the analysis of chromosomes was taken, with little alterations, from work by Moorehead et al. (1960). A mouse bearing the ascites tumor cells was given a subcutaneous injection of colchicine (0.2 mg. per 100 gm. body weight) and killed two hours

later. The ascitic fluid was removed and treated as outlined in Moorehead's paper. The cells were stained with aceto-orcein and the chromosomes of over four hundred cells were counted.

TRANSFER OF TUMORS

Melanoma: Donor mice containing the B16 melanoma were killed by cervical dislocation on days 12 or 14 after transplantation. The melanoma was removed from the donor animal: non-necrotic portions were placed in a petri dish containing Hank's balanced salt solution (pH 7.2 - 7.6) and cut into small pieces with scalpel blades.

The experimental mice were lightly anaesthetized and a small incision was made in the lower right abdomen. An alcohol cleaned probe was then placed in the incision to separate the skin from the peritoneal wall and three or four small pieces of melanoma pushed into the pocket thus formed. The incision was closed with a 11 mm. Michel wound clip. No infection at the site of transplantation was seen in any of the animals. The day of tumor transplantation was designated as day 0 of tumor growth.

Ascites: A group of mice were maintained only to provide an available tumor pool. On day 7, after receiving an innoculum of ascites fluid, these mice were killed by chloroform anaesthesia or cervical dislocation. The abdomen of the donor animal was washed with 70 percent alcohol and the ascites fluid was removed from the abdominal cavity by means of a $2\frac{1}{2}$ cc. plastic disposable syringe with a 25 gauge 5/8 inch needle. The ascites fluid was then injected directly, undiluted in 0.2 cc. amounts, into the experimental animals. The day on which the experimental mice

received the ascites innoculum was designated as day 0.

ADMINISTRATION OF COLCHICINE

Colchicine was used in experiments M1, M2, M3, M6, M7, M8, A1, A2, A3, and A6. All mice, both in experimental and control groups, received an interscapular, subcutaneous injection of colchicine (0.2 mg. per 100 gm. body weight) four hours prior to being killed. The colchicine was obtained from Inland Alkaloid Inc., Tipton, Indiana.

ADMINISTRATION OF TRITIATED THYMIDINE

Tritiated thymidine (3H-T) of 5 c/mM specific activity was used in experiments M4 and M5 and A4 and A5. All mice received a single intraperitoneal injection of ^{3}H-T (25μC/ 0.25 ml of distilled water). In experiments M4 and A4, pulse labels of tritiated thymidine were given 15 minutes before killing the animals. However, ^{3}H-T was given at 10:00 a.m. on the 10th day of experiment M5 and at 10:00 a.m. on the 6th day of experiment A5. The ^{3}H-T was obtained from the New England Nuclear Corp., Boston, Mass.

DRUG USED

Ara-C (U - 19,920: Lot no. 8240 - BDA - 120) was received as a gift from the Upjohn Company (Kalamazoo, Michigan), through the courtesy of Dr. Gunther S. Fonken.

Ara-C has been shown to be stable in water for approximately seven days if stored at refrigerated temperatures (Livingston and Carter, 1968).

ADMINISTRATION OF ARA-C

In all experiments ara-C was dissolved in distilled water, at different concentrations and injected intraperitoneally in 0.2 cc. doses.

It is important to note that each experiment begins when the tumors are transferred and this was designated as day 0. Day 0 began at 10:00 a.m. and lasted for 24 hours until 10:00 a.m. the following day which was the beginning of day 1.

Experiment No: M1

The purpose of this experiment was to determine the daily mitotic rates of B16 melanoma after the treatment of 50 and 80 mg/kg dosages of ara-C.

In this experiment, 48 JAX mice bearing the B16 melanoma tumor received a single injection of ara-C at 10:00 a.m. on the tenth day after tumor transplantation. Half (24) of this group received 50 mg/kg of ara-C while the other half (24) received 80 mg/kg of ara-C. Another 24 tumor bearing mice served as untreated controls. The average weight of the melanoma bearing mice was 22 gms.

These animals were further divided into 6 subgroups of 12 animals, each subgroup containing 4 animals from each experimental group and 4 from the control group. All animals were injected with colchicine on the tenth day after transplantation and killed 4 hours later. Injection of animals was arranged so that each of the 6 groups were killed at 2:00 p.m., 6:00 p.m., 10:00 p.m., 2:00 a.m., 6:00 a.m., and 10:00 a.m. The summation of the mitotic rates in each of the experimental groups and the

control group equalled the number of cells being added to the total tumor population over a 24 hour period. These values represented the daily mitotic rate of the melanoma tumors for the treated groups and the control group.

Experiment No: M2

The aim of this experiment was to determine the effect of 2 and 3 single daily injections of ara-C (50 mg/kg) upon the mitotic rate of the B16 melanoma.

In part I four melanoma-bearing JAX mice received a single injection of ara-C (50 mg/kg) at 10:00 a.m. on the 10th and 11th days after tumor transplantation. Immediately after the last injection on day 11, the treated and control groups received an injection of colchicine and subsequently were killed at 2:00 p.m.

In part II, the same procedure was carried out, with daily injections on days 10, 11 and 12 at 10:00 a.m. Both treated and control groups received colchicine at 10:00 a.m. on day 12 and were killed four hours later. In both parts I and II, the four hour mitotic rates of the melanoma tumor (10:00 a.m. to 2:00 p.m.) were determined for both treated and control groups.

Experiment No: M3

The purpose of this experiment was to determine the effect of a number of single daily injections of ara-C (50 mg/kg) upon the transplantation of the B16 melanoma.

A JAX melanoma-bearing mouse received one (50 mg/kg) injection of

ara-C at 10:00 a.m. on the 10th day of tumor growth. A second melanoma bearing mouse received a daily injection of ara-C, same dosage, at 10:00 a.m. on days 10 and 11, while a third melanoma bearing mouse received a single daily injection of ara-C at 10:00 a.m. on days 10, 11 and 12 of tumor growth.

Four hours after the last injection of ara-C, each donor animal was killed and its melanoma tumor was transplanted into a group of four non-tumor bearing mice. The tumors, in each group, were allowed to grow for ten days. Each group received an injection of colchicine at 10:00 a.m. on the tenth day of tumor growth and were killed four hours later. The mitotic rate of the B16 melanoma was determined for each group.

Experiment No: M4

The aim of this experiment was to determine the effects of a single injection of ara-C (50 mg/kg) upon the thymidine index of B16 melanomas.

Forty-four melanoma-bearing JAX mice each received a single injection of ara-C (50 mg/kg) at 10:00 a.m. on the 10th day of tumor growth. The first treated group (four animals) received an injection of tritiated thymidine immediately after the ara-C injection, and was killed 15 minutes later. The second group was killed 30 minutes after the injection of ara-C and 15 minutes after an injection of tritiated thymidine. Other groups were killed 45 minutes, 1, 2, 4, 8, 12, 16, 20 and 24 hours after the injection of ara-C and in all instances received a ^{3}H-T injection 15 minutes prior to death. Control groups (each of two mice) were killed 15 minutes after receiving tritiated thymidine at the 30 minute, 1, 12 and 24 hour intervals.

The thymidine index represents the number of cells undergoing DNA synthesis during the 15 minute period in which tritiated thymidine was available before the animals were killed.

Experiment No: M5

The purpose of this experiment was to determine the effect of a single injection of ara-C upon the cell cycle of the B16 melanoma.

Ninety-six tumor-bearing JAX mice received a single injection of tritiated thymidine at 10:00 a.m. on the 10th day of tumor growth. Fifty-six of these mice were divided into 14 groups of 4 animals each and all received a single 50 mg/kg injection of ara-C at 11:00 a.m. on the same day. Beginning one hour later, that is at 12:00 noon, a group of four treated and a group of two untreated mice were killed. Only two mice per control group were used due to expense. The second groups of treated and control mice were killed three hours after the injection of ara-C. The remaining groups, treated and control, were killed at 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, and 27 hours after the injection of ara-C.

The percent labeled mitoses were plotted, for both groups, against time.

Experiment No: M6

The aim of this experiment was to determine the effect of four injections of ara-C (50 mg/kg) on the mitotic rate of B16 melanoma on days 6 and 10 of tumor growth.

On the 6th day of tumor growth, five groups, each of five melanoma bearing mice, were given four injections of ara-C, at eight hour intervals, that is at 10:00 a.m., 6:00 p.m., 2:00 a.m., and 10:00 a.m. The

first group of treated mice was given colchicine at 2:00 p.m. and killed at 6:00 p.m. on the 6th day. The second group was given colchicine at 6:00 a.m. on day 9 and killed at 10:00 a.m. the beginning of day 10. The remaining three groups received four more injections of ara-C on day 10, following the same schedule used on day 6. This 3 day rest period allowed the animals to overcome any toxic effects of ara-C (Skipper et al., 1967). The third group was given colchicine at 2:00 p.m. and killed at 6:00 p.m. on day 11. The fourth group on the third day of the second host recovery period was given colchicine at 6:00 a.m. (day 13) and killed at 10:00 a.m. the beginning of day 14. During this host recovery period eight animals died and had to be discarded from the experiment.

The mitotic rates were determined for these groups.

Experiment No: M7

The purpose of this experiment was to determine the effect of four injections of ara-C (25 mg/kg), on the mitotic rate of the B16 melanoma after 6, 8, and 10 days of tumor growth.

Six groups, each of four melanoma-bearing mice, were given four injections of ara-C on day six of tumor growth, at eight hour intervals, that is, at 10:00 a.m., 6:00 p.m., 2:00 a.m., and 10:00 a.m. This same procedure was carried out on days 8 and 10 of tumor growth. An interval of one day was allowed between the injection series of ara-C to allow for host recovery. The first group of treated mice was given colchicine at 2:00 p.m. and killed at 6:00 p.m. on day 7 of tumor growth, thus the mice were killed eight hours after the last injection of ara-C.

The second group was given colchicine at 6:00 a.m. on day 7 of tumor growth and killed at 10:00 a.m. The third, fourth, fifth and sixth groups received four more injections of ara-C on day 8 of tumor growth. The third group was killed eight hours after the last ara-C injection while the fourth group was killed 24 hours after the last injection. The fifth and sixth groups received four more ara-C injections on day 10 of tumor growth. The fifth group was killed eight hours after the last injection of ara-C and four hours after a colchicine injection. The sixth group did not survive through the third host recovery period, and had to be discarded from the experiment.

The mitotic rates were determined.

Experiment No: M8

The aim of this experiment was to determine the effect of four injections of ara-C (12.4 mg/kg) on the mitotic rate of B16 melanoma on days 6, 8 and 10 of tumor growth. Six groups, each of four melanoma-bearing mice, were given four injections of ara-C on day 6 of tumor growth, at eight hour intervals, that is 10:00 a.m., 6:00 p.m., 2:00 a.m., and 10:00 a.m. The same procedure was followed in the experiment as was previously described in experiment number M7. Colchicine was injected in all cases four hours before the group was killed. No animals died in this experiment. The mitotic rates were determined.

Experiment No: A1

The aim of this experiment was to determine the daily mitotic rate of Ehrlich ascites tumor cells after injection of a 50 mg/kg dose of ara-C.

In this experiment 24 Connaught mice bearing EAT cells, received a single 50 mg/kg injection of ara-C at 10:00 a.m. on the 6th day after tumor transplantation. Another 24 tumor bearing mice served as untreated controls. The average weight of the ascites-bearing mice was 35 gm.

The first group, consisting of four animals in each of the treated and untreated control series, received an injection of colchicine at 10:00 a.m. on the 6th day after transplantation. These two groups were killed at 2:00 p.m., that is four hours later. The second group in each series of treated and control animals received colchicine at 2:00 p.m. and were killed at 6:00 p.m. The same procedure was carried out for the remaining four groups in each series, and were killed at 10:00 p.m., 2:00 a.m., 6:00 a.m., and 10:00 a.m., respectively. The summation of the six, 4-hour groups in each of the treated and untreated control series equalled the number of cells added to the total tumor population over a 24-hour period. These percentages represented the daily mitotic rate of EAT cells for the treated and control series of animals.

Experiment No: A2

The purpose of this experiment was to determine the effect of 2 and 3 single daily injections of ara-C (50 mg/kg) upon the mitotic rate of Ehrlich ascites tumor cells.

In part I, four EAT-bearing Connaught mice received a single (50 mg/kg) injection of ara-C at 10:00 a.m. on the 6th and 7th days after tumor transplantation. Immediately after the last injection on day 7, the treated and untreated control groups received an injection of colchicine and subsequently were killed at 2:00 p.m.

In part II, the same procedure was carried out, with daily injections at 10:00 a.m. on days 6, 7, and 8. Both treated and untreated control groups received colchicine at 10:00 a.m. and were killed four hours later on day 8. In both parts I and II, the four-hour mitotic rates of EAT cells (10:00 a.m. - 2:00 p.m.) were determined for both treated and control groups.

Experiment Number A3

The purpose of this experiment was to determine the effect of a number of single daily injections of ara-C (50 mg/kg) upon the transplantation of the EAT cells.

A Connaught EAT-bearing mouse received one (50 mg/kg) injection of ara-C at 10:00 a.m. on the 6th day of tumor growth. A second EAT-bearing mouse received the same dose of ara-C, at 10:00 a.m. on both the 6th and 7th days, while a third EAT-bearing mouse received a single daily injection of ara-C at 10:00 a.m. on the 6th, 7th, and 8th days of tumor growth.

Four hours after the last injection of ara-C, each donor animal was killed and its EAT cells were innoculated into a group of four non-tumor bearing mice. The EAT cells in each group were allowed to grow for six days. Each group received an injection of colchicine at 10:00 a.m. on the sixth day of tumor growth and were killed four hours later. The mitotic rate of the EAT cells was determined for each group.

Experiment No: A4

The aim of this experiment was to determine the effects of a single 50 mg/kg injection of ara-C upon the thymidine index of EAT cells.

Forty-four EAT-bearing mice received a single 50 mg/kg injection of ara-C at 10:00 a.m. on the 6th day of tumor growth. The first treated group, having four animals, received an injection of tritiated thymidine immediately after the ara-C injection, and was killed 15 minutes later. The second group was killed 30 minutes after the injection of ara-C and 15 minutes after an injection of tritiated thymidine. Other groups were killed 45 minutes, 1, 2, 4, 8, 12, 16, 20, and 24 hours after the injection of ara-C, and in all instances received $T\text{-}H^3$ 15 minutes prior to death. Control groups, each of two mice, were killed 15 minutes after receiving tritiated thymidine, at the 30 minute, 1, 12, and 24 hour intervals.

The thymidine index represents the number of cells undergoing DNA synthesis during the 15 minute period in which tritiated thymidine was available.

Experiment No: A5

The purpose of this experiment was to determine the effect of a single injection of ara-C upon the cell cycle of EAT cells.

Ninety-six tumor bearing Connaught mice received a single injection of tritiated thymidine at 10:00 a.m. on the 6th day of tumor growth. Fifty-six of these mice were divided into 14 groups of four animals each, and all received a single 50 mg/kg injection of ara-C at 11:00 a.m. on the same day 6. Beginning one hour after the ara-C injection, a treated group and a non-treated control group were killed. The non-treated control group was made up of only two animals as an economy measure. The

second group of treated and untreated control animals were killed three hours after the injection of ara-C. The remaining groups, both treated and untreated, were killed at 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, and 27 hours after the single injection of ara-C.

The cell cycle for both was plotted.

Experiment No: A6

The aim of this experiment was to determine the effect of four injections of ara-C (25 mg/kg) on the mitotic rate of EAT cells on the third, 5th and 7th days of tumor growth.

Seven groups of four EAT-bearing mice were given four injections of ara-C on the 3rd day of tumor growth, at eight hour intervals, ie., at 10:00 a.m., 6:00 p.m., 2:00 a.m., and 10:00 a.m. This same procedure was carried out on days five and seven of tumor growth. An interval of one day was allowed between each series of injections to allow for host recovery. The first group of treated mice was given colchicine at 2:00 p.m. and killed at 6:00 p.m. on day four of tumor growth, thus the mice were killed eight hours after the last injection of ara-C. The second group was given colchicine at 6:00 a.m. on day four and killed at 10:00 a.m. The remaining groups received four more ara-C injections on day 5 of tumor growth. Group three was killed eight hours after the last injection of ara-C while the fourth group was killed 24 hours after the last injection. The fifth, sixth and seventh groups received four more ara-C injections on day 7 of tumor growth. The fifth group was killed eight hours after the last injection of ara-C, while

the sixth group was killed 24 hours after the last ara-C injection. All groups received colchicine four hours before being killed. The seventh group did not survive the last injection series on day 7 and had to be discarded from the experiment.

Killing and Histological Technique

All mice were killed by cervical dislocation. Tumors of mice in experiments M1, M2, M3, M6, M7, M8, A1, A2, A3, and A6 were fixed in Davidson's fixative. Tumors of mice in experiments M4 and M5 and A4 and A5, that is those used for making autoradiographs, were fixed in buffered formalin.

The ascites tumor cells were absorbed into small squares (approximately 5 $mm.^2$) of Gelfoam (The Upjohn Co., Kalamazoo, Michigan). This method was previously described by Leighton et al. (1957) and Bertalanffy et al. (1965). The Gelfoam blocks containing the ascites cells were fixed in either Davidson's or buffered formalin, depending on whether colchicine or ^{3}H-T was used. The melanoma tumor was dissected out and cut into small pieces and placed in Davidson's or buffered formalin, depending on whether the colchicine or ^{3}H-T technique was used.

The tissues and sponges to be used in the colchicine method were cut at 7μ. Those to be used for autoradiograph techniques were cut at 3 - 4 μ. Every tenth section was placed on a glass slide and all sections were stained with hematoxylin and eosin.

Autoradiographic Technique

The technique used was suggested by Kopriwa (1967). Kodak's NTB-3

type liquid emulsion and D-19 developer were used.

Autoradiographic Technique Steps

1. Tissues were cut at 3 - 4 μ and placed on alcohol (70%) cleaned slides.
2. Deparaffinization of tissues:
   a) two changes of xylol (five minutes each).
   b) two changes of absolute alcohol (three minutes each).
   c) 95% alcohol (three minutes).
   d) 70% alcohol (three minutes).
   e) distilled water (three minutes).
3. Coating of slides with emulsion:*

   The emulsion was melted in a water bath (40^{0}C) for one to one and one-half hours. The slides were placed into the emulsion for one second and removed. They were then placed on a rack and dried in an incubator, kept at a relative humidity of over 70 percent and a temperature of 28^{o}C for one hour. When dry, the slides were placed in a black slide box. A small gauze sac of "Dririt" was placed in the base of the slide box. Slide boxes were sealed with heavy black tape and stored with the tissues facing upward.
4. Slides were exposed for 10 to 12 days.
5. Developing procedure:*
   a) D-19 (six minutes).
   b) distilled H_2O (10 dips).

c) Kodak fixer (10 minutes).

d) rinsed in gently running tap water (10 minutes).

6. Staining procedure:

a) hematoxalin (time variable)**

b) tap water (two minutes)

c) acid alcohol (three quick dips)

d) tap water (two minutes)

e) lithium carbonate (two minutes)

f) tap water (two minutes)

g) eosin (0.2% alcohol) (one minute)

h) 70% alcohol (five dips)

i) 95% alcohol (two minutes)

j) two changes absolute alcohol (two minutes each)

k) two changes xylol (two minutes each)

7. Permanent slide preparations were made using Permount.

* these procedures were done in the dark.

** melanotic tissue - 3 minutes; ascites tumor cells one half minute.

Enumeration of Nuclei

All sections which had been treated with colchicine were examined with a binocular light microscope at a magnification of 400X, while autoradiographic slides were viewed under an oil immersion objective at 1000X. A micrometer disc (Bausch and Lomb) was used to define the area to be counted. The selection of the fields to be counted was random except that in the case of the B16 melanoma tumors, counting was confined

to non-necrotic areas. A two-column tally counter was used to record the counts. In the experiments where colchicine was employed, over 4,000 cells per animal were counted and recorded as either an interphase or colchicine metaphase cell. Prophases were included in the interphase count, while no anaphase or telophase cells were seen. Colchicine metaphases were recognized by the following features although not all of these necessarily appeared together. 1) darkly staining clumps of chromatin, 2) a peripheral halo of cytoplasm, 3) a swollen cell outline, and 4) absence of the nuclear membrane (Wallace, 1964). The majority of the colchicine nuclei had these characteristics although some difficulty was experienced in distinguishing them from nuclei exhibiting pyknosis or karyorrhexis.

In those sections which had undergone the autoradiographic technique, a cell was considered labeled if three or more silver grains appeared above the nucleus. In most cases, labeled cell nuclei were well labeled with many grains. In experiments M4 and A4, over 1,000 cells were counted and classified as either labeled or unlabeled. In experiments M5 and A5, a minimum of 100 mitoses were counted in the untreated series as either labeled or unlabeled. In the treated series, fewer mitoses could be found, especially in the B16 melanoma.

For determining the duration of the mitotic phase for both the B16 melanoma and EAT cells the criteria outlined by Leblond and Stevens (1948) for the various stages of mitosis were used.

> "Prophase was taken to have started when definite chromatin filaments were arranged along the nuclear membrane in spoken wheel fashion. Later, the prophase nucleus became

more swollen, the chromatin threads became thicker and there was a gradual disappearance of the nuclear membrane and nucleolus.

"Metaphase was easily distinguished as the stage in which no trace of the membrane was visible. The chromosomes were arranged in a central clump. The cytoplasm usually appeared paler and slightly granular.

"Anaphase was diagnosed as lasting from the first separation of two chromosome clumps moving towards the two poles, up to the first appearance of a mid-plate dividing the cytoplasm into two daughter cells.

"Telophase extended from the last stage of anaphase to one which merged with normality."

Statistical Methods

The mean, standard errors and the F values of the data were obtained from the Factorial analysis of variance (MCST12), The University of Manitoba, Computer Centre. A "Student-t" test was used to compare means, in which $n_1 = n_2$ (Gray, 1961).

A five percent level of significance was used to accept or reject the nul hypothesis.

CHAPTER IV.

RESULTS

The purpose of these experiments was to determine the effect of ara-C upon the cell proliferation of the B16 melanoma and Ehrlich ascites tumor cells.

In the fall of 1967 a study of the possible action of ara-C upon cells about to undergo DNA synthesis was presented to the author. In order to test the action of ara-C upon such cells, a number of pilot experiments were performed, and their observations are presented in the following, which serves as an introduction to the main part of the work.

Three groups of Swiss R male mice were injected subcutaneously with 0.833 mg. of ara-C / 0.1 ml. of distilled water every four hours over a 72 hour period. The first group of mice was killed after 24 hours, and had received a total of 5 mg. of ara-C. The second group was killed 48 hours after receiving a total dosage of 10 mg. of ara-C, while the third group was sacrificed 72 hours later and had received 15 mg. of ara-C. The animals of all groups were suffering from the toxic effects of the drug. Samples of duodenum from each animal were fixed in Davidson's fixative, cut at 7 μ and routinely stained with hematoxylin and eosin. The intestinal epithelium of the 24-hour group appeared normal but did not exhibit any mitotic figures, which normally occur in great abundance, Photo 1. The intestine of the second group were abnormal, Photo 2. The villi appeared shorter than usual and the epithelial cells covering the villi were greatly enlarged. This enlargement appeared to be an adaptive process because fewer epithelial cells covered the villi after the prolonged treatment with ara-C. Moreover, only remnants of the crypts

of Lieberkühn remained, and a chronic lymphocytic infiltrate was present. As indicated in Photo 3, 72 hours after ara-C (15 mg.) treatment, the villi had all but disappeared and the epithelial lining was discontinuous. The animals subjected to such treatment were extremely ill and this can presumably be ascribed to a loss of absorptive ability of the intestine, and lack of water retention. Similar observations were published recently by Leach et al. (1969). It might be interesting to mention that 5, 10, and 15 mg. dosages of ara-C administered to rats apparently produced no effect at all on the intestinal mucosa of Sprague-Dawley rats, the drug appearing to be ineffective in that species. This phenomenon was previously observed by Camiener and Smith (1965).

Experiment No: M1

The purpose of this experiment was to determine the daily mitotic rates of B16 melanoma after treating tumor-bearing mice with dosages of 50 and 80 mg./kg. of ara-C on the tenth day after tumor transplantation.

The six, four-hour mitotic rates obtained from melanoma tumor cells after treatment with 50 and 80 mg/kg doses of ara-C, respectively, are compared to those of untreated control groups in Table 1, and graphically illustrated in Figure 4.

a) In the tumors of untreated animals, the four hourly mitotic rates varied significantly during the 24 hour period, the minimum being 5.12 percent in the 6:00 p.m. - 10:00 p.m. group and the maximum 10.02 percent in the 2:00 a.m. - 6:00 a.m. group. The daily mitotic rate was determined by addition of the six 4-hour groups, and was found to

TABLE 1

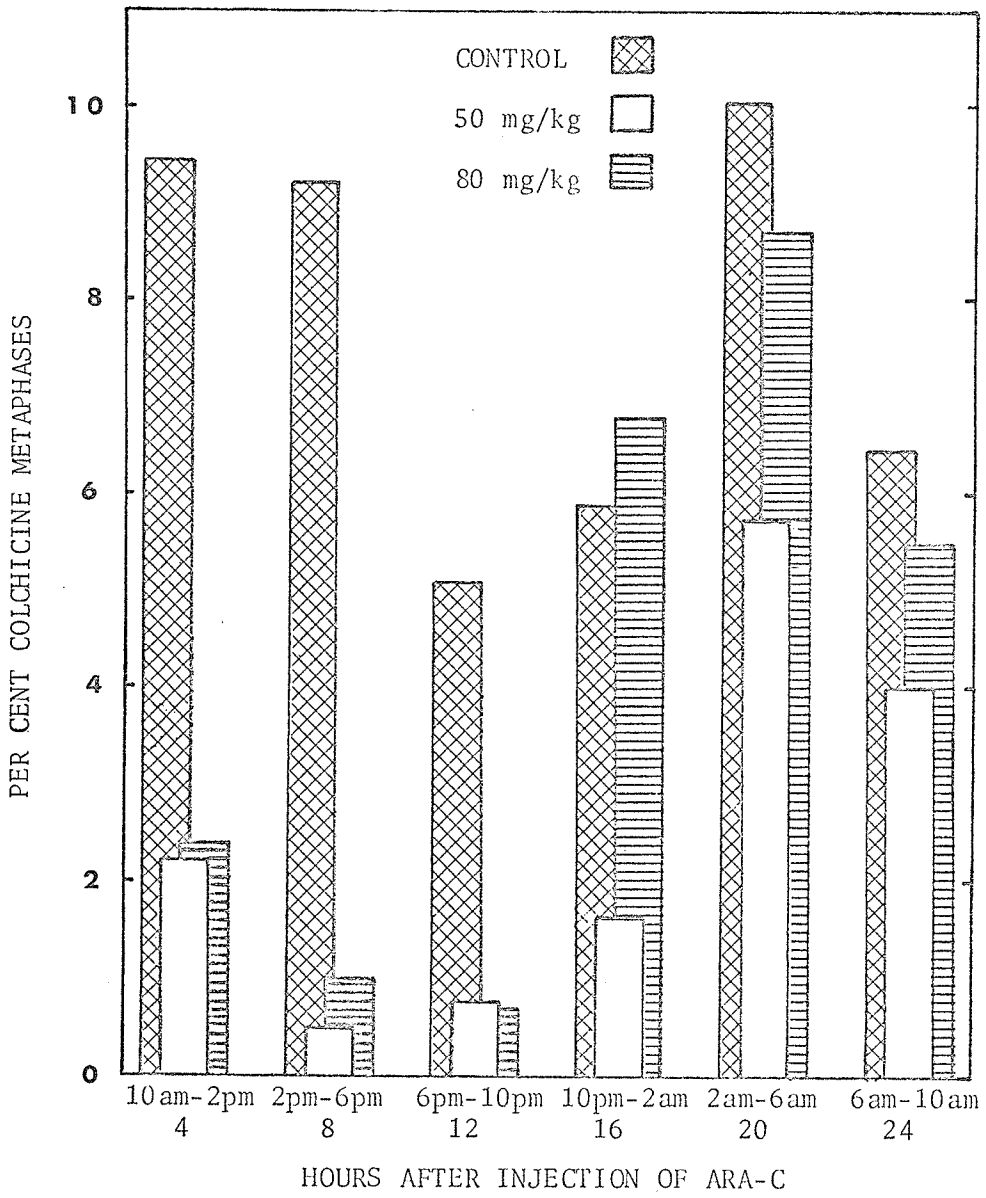
DAILY MITOTIC RATE OF B16 MOUSE MELANOMA ON DAY 10
AFTER A SINGLE 50mg/kg AND 80mg/kg INJECTION OF ARA-C

DOSAGE OF ARA-C (mg/kg)	TIME OF DAY	NUMBER OF NUCLEI	NUMBER OF METAPHASES	PERCENTAGE METAPHASES	DAILY MITOTIC RATE
CONTROL	10am-2pm	17,359	1,639	9.47 ± 0.90	
	2pm-6pm	17,369	1,595	9.20 ± 1.71	
	6pm-10pm	17,195	884	5.12 ± 0.90	
	10pm-2am	17,224	1,026	5.90 ± 1.17	
	2am-6am	17,396	1,733	10.02 ± 1.58	
	6am-10am	18,130	1,167	6.43 ± 0.37	46.14 ± 6.63
50	10am-2pm	16,443	370	2.24 ± 0.16	
	2pm-6pm	16,104	91	0.56 ± 0.04	
	6pm-10pm	16,299	128	0.78 ± 0.31	
	10pm-2am	16,515	275	1.64 ± 0.58	
	2am-6am	16,492	950	5.75 ± 1.01	
	6am-10am	12,342*	494	4.00 ± 0.31	14.97 ± 2.41
80	10am-2pm	17,616	424	2.41 ± 0.33	
	2pm-6pm	17,331	182	1.04 ± 0.06	
	6pm-10pm	16,332	114	0.69 ± 0.10	
	10pm-2am	17,447	1,204	6.81 ± 1.68	
	2am-6am	16,847	1,470	8.70 ± 0.80	
	6am-10am	18,279	1,008	5.53 ± 0.63	25.18 ± 3.60

* one animal died.

FIGURE 4

THE MITOTIC PATTERN OF 50mg/kg AND 80mg/kg ARA-C TREATED AND CONTROL B16 MELANOMA TUMOR CELLS



be 46.14 $\pm$ 6.63 percent. The doubling time of the tumor, that is the time required for 100 percent of the tumor cells to divide, is thus about 52 hours.

b) In the tumors of mice treated with 50 mg/kg of ara-C, there was likewise a significant range of variability between the six 4-hour groups. In the first group of mice, killed after four hours of ara-C action, the mitotic rate had declined from the normal 9.47 percent to 2.24 percent. This represented a 75 percent reduction in the mitotic rate within the first four hours. A further decrease occurred in the second group after 8 hours attaining a low of 0.56 percent. The mitotic rates of the subsequent groups, that is, after 12, 16, 20 and 24 hours of ara-C action, began to increase progressively. Yet in no instance was the level of the mitotic rate of the untreated tumors reached. Addition of the six 4-hour mitotic rates signified that the daily mitotic rate was reduced to 14.97 $\pm$ 2.41 percent by the ara-C treatment. This represented a depression of the daily mitotic rate of approximately 66 percent by the drug.

c) In the tumors of mice, treated with 80 mg/kg of ara-C, a statistically significant variability was seen between the six 4-hour groups. The mitotic rate of the tumors from the first group of mice, which were killed four hours after the administration of ara-C, was 2.41 percent. This represented a reduction of approximately 74 percent over normal during the first four hours. The mitotic rate became furthur reduced to 1.04 and 0.69 percent, 8 and 12 hours respectively after

the administration of ara-C. In the tumors of the 16-hour group, however, a complete recovery was noted, and the mitotic rates of those of the 20-hour and 24-hour groups were comparable to those of the control groups. The daily mitotic rate achieved with the 80 mg/kg dose of ara-C was 25.18 $\pm$ 3.60 percent, and showed a reduction in mitotic rate of the tumors of about 50 percent. The decrease in mitotic rates four hours after ara-C administration with both the 50 and 80 mg/kg dosages were not significantly different; the same was true for the 12 hour groups. However, the differences between the mitotic rates of all subsequent groups were highly significant ($p < 0.01$). In all instances the differences between the mitotic rates of control and treated animals was also highly significant ($p < 0.01$).

It will be noted that the 50 mg/kg dose of ara-C was more effective in reducing mitotic activity than the 80 mg/kg dose. As a consequence, this was used in all subsequent experiments.

Experiment No: M2

The purpose of this experiment was to determine the effect of two and three serial injections of ara-C, given at daily intervals, upon the mitotic rate of the B16 melanoma.

The percentage metaphases arrested in the tumors during the 4-hour periods of mice receiving two or three injections of the drug are compared with those of the untreated control groups in Table 2. The tumors of the treated group, receiving 2 injections of ara-C, exhibited a mitotic rate of 1.61 percent as compared with the mitotic rate of 6.37 percent

found in the control group. A mitotic rate of 1.18 percent was found in the second treated group, which received three injections of ara-C, while that of the untreated control group was 4.97 percent.

The difference between the mitotic rates of tumors treated with both two and three doses of ara-C was found to be significant, although three injections of ara-C produced a more pronounced depression of the mitotic rate than either one (taken from the first 4-hour 50 mg/kg treated group of experiment No: M1) or two injections.

Experiment No: M3

The purpose of this experiment was to ascertain whether ara-C would affect the transplantation of B16 melanoma cells. The mean mitotic rate of tumors, which had been transplanted from a donor animal receiving a single injection of ara-C, was 7.55 percent (Table 3). The mitotic rates of tumors transplanted from donor mice receiving two or three injections of ara-C, were 5.17 and 6.13 percent, respectively. All these values were determined 10 days after transplantation. The reduction of mitotic activity by pretreatment with 2 injections of ara-C was highly significant ($p < 0.01$), while the effects of one and three injections were not significant.

Experiment No: M4

The aim of this series was to demonstrate the effect of a single injection of ara-C upon the thymidine index (the percentage of tumor cells labeled by a single injection of tritiated thymidine) of the B16 melanoma. Groups of tumor-bearing mice were killed at different time intervals after

TABLE 2

EFFECT OF 2 AND 3 INJECTIONS OF ARA-C (50mg/kg)
ON THE MITOTIC RATE OF B16 MELANOMA

NUMBER OF INJECTIONS	ON DAYS	NUMBER OF NUCLEI	NUMBER OF METAPHASES	PERCENTAGE OF METAPHASES
2	10,11	17,382	279	1.61 ± 0.28
CONTROL		16,669	1,067	6.37 ± 1.31
3	10,11,12	16,716	198	1.18 ± 0.17
CONTROL		16,716	1,032	4.97 ± 0.74

TABLE 3

TRANSPLANTABILITY OF B16 MELANOMA TUMOR CELLS
AFTER 1, 2 AND 3 INJECTIONS OF ARA-C (50mg/kg)

NUMBER OF INJECTIONS	NUMBER OF NUCLEI	NUMBER OF METAPHASES	PERCENTAGE OF METAPHASES
1	16,349	1,236	7.55 ± 0.50
2	17,318	893	5.17 ± 0.74
3	16,716	1,032	6.13 ± 1.38

ara-C administration, having first received a pulse label of tritiated thymidine 15 minutes prior to sacrifice. The thymidine index was determined for four untreated control groups, 10 days after tumor transplantation, at ½, 1, 12, and 24 hour intervals, and these are compared with tumor specimens from animals receiving ara-C treatment. The thymidine index of the treated animals was determined at more frequent intervals (Table 4). The data are graphically plotted against time in Figure 5.

In Figure 5, a straight line was drawn between the control values at 13.50 percent, taking this to be the mean labeling level of the untreated tumors. In the treated tumor specimens, the thymidine index decline from the normal value to 2.08 percent within 15 minutes after pulse labelling. This low level was maintained and reached a minimum of 0.21 percent at one hour after ara-C administration. Subsequently, the proliferative activity of the cells as expressed by the thymidine index, began to recover from the action of ara-C, and by 12 hours had reached the normal range of the control specimens. This was followed by an "overshoot" of the thymidine index at the 16-hour point, exceeding the normal mean level, but the large standard error ($\pm$ 5.52) should be noted. A slight decline was subsequently noted, so that the indices of the 20 and 24 hour periods were slightly below the control level.

Experiment No: M5.

The purpose of this experiment was to determine the effect of ara-C treatment on the cell cycle of the B16 melanoma. The normal percentage

TABLE 4

EFFECT OF A SINGLE INJECTION OF ARA-C (50mg/kg) UPON THE THYMIDINE INDEX OF B16 MELANOMA TUMOR CELLS ON DAY 10

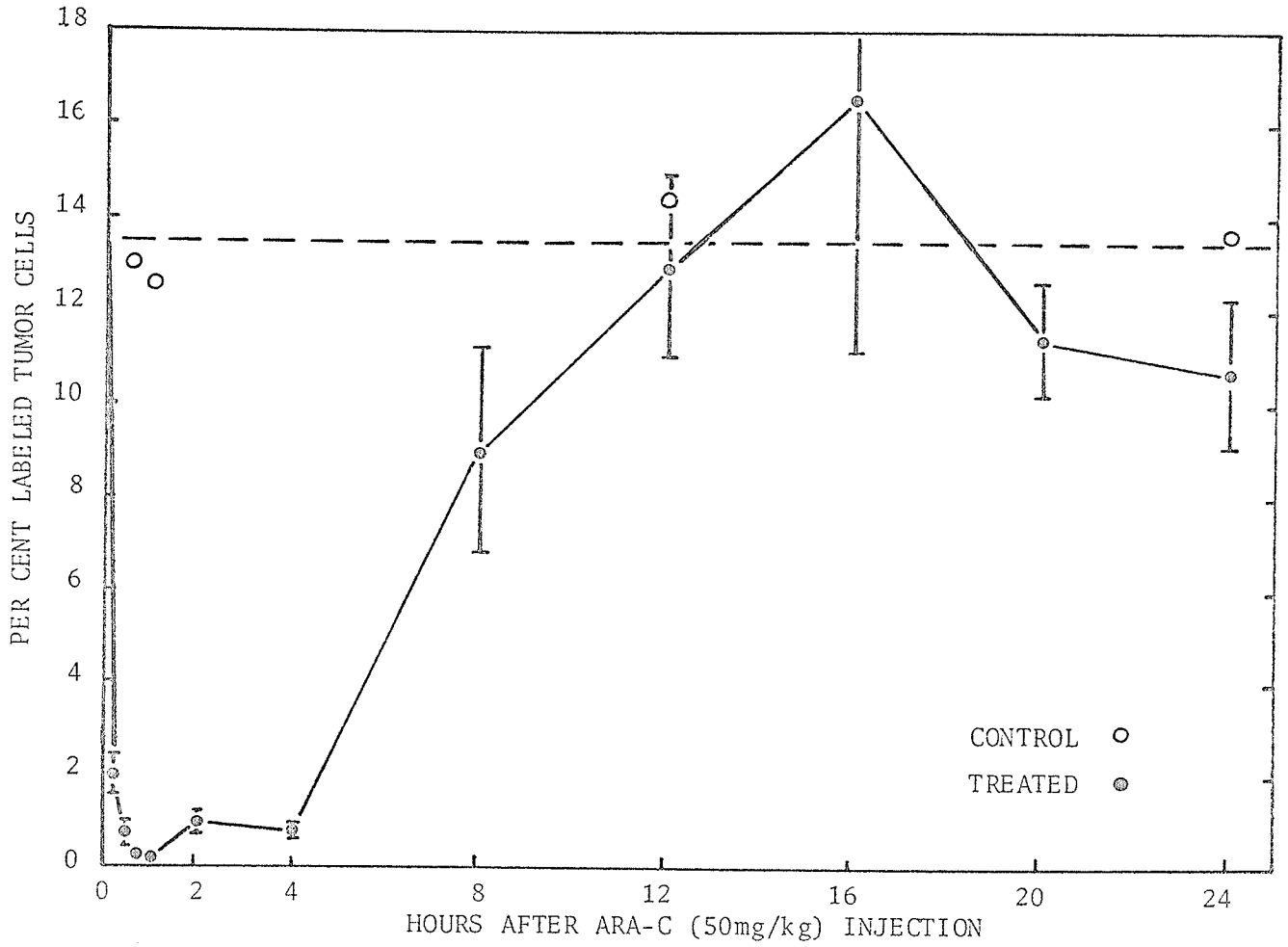
HOURS AFTER INJECTION OF ARA-C	TOTAL NUMBER OF CELLS	NUMBER OF LABELED CELLS	THYMIDINE INDEX (%)
0.25	4,024	84	2.08 ± 0.43
0.50	3,016*	20	0.66 ± 0.17
(0.50)	(2,026)	(265)	(13.07)
0.75	4,026	10	0.24 ± 0.05
1	4,224	9	0.21 ± 0.05
(1)	(2,028)	(255)	(12.57)
2	4,102	42	1.02 ± 0.26
4	4,211	35	0.82 ± 0.17
8	4,137	368	8.91 ± 2.22
12	4,211	548	12.94 ± 1.89
(12)	(2,116)	(305)	(14.38)
16	4,343	703	16.55 ± 5.52
20	4,093	467	11.41 ± 1.24
24	4,242	454	10.74 ± 1.66
(24)	(2,075)	(285)	(13.71)

* one mouse died.

( ) control groups with two mice each.

FIGURE 5

THYMIDINE INDEX OF B16 MELANOMA CELLS AT VARIOUS INTERVALS AFTER THE INJECTION OF ARA-C



of labeled mitoses (Table 5) was plotted against time after tritiated thymidine injection (Figure 6). The cell cycle time (t_C) of untreated melanoma cells was estimated from these data to be 14 hours, with a minimum duration of the S-phase of 7.5 hours. The interval of the pre-mitotic gap (G_2) and one-half of the duration of mitosis was estimated to last 2 hours. The duration of mitosis was ascertained in tumor bearing mice (using the formula of Stevens-Hooper) to be 1 hour and 21 minutes (Table 6). From these data, the length of the G_1 phase was determined to be 3 hours and 50 minutes by the following calculation: G_2 = 2 hrs - $\frac{1.21}{2}$ or 1.21 ÷ 2 = 1 hr. and 19 min., and the post-mitotic phase $G_1 = t_C - (G_2 + M + S)$ or 14 hrs. - (1 hr. 19 min. + 1 hr. 21 min. + 7 hrs. 30 min.) = 3 hours and 50 minutes.

The percentage of labeled mitoses from the ara-C treated tumors (Table 7) were plotted against time, following the injection of tritiated thymidine (Figure 7). The drug effect became evident as the ascending limb of the first curve began but soon leveled off at about the 36 percent level. Seven hours after ara-C treatment, the percentage of labeled mitoses declined to 14.68, and subsequently increased sharply after 9 hours to 67.76 percent. A peak of labeled mitosis was attained 11 hours after ara-C treatment, at 74.14 percent. The percentage of labeled mitoses declined subsequently, before rising at 26 or 28 hours to start a second peak. The duration of the only phase which could be determined, was the S-phase, equalling about 6 hours.

Experiment No: M6 The aim of this experiment was to ascertain

TABLE 5

CELL CYCLE OF UNTREATED B16 MOUSE MELANOMA ON DAY 10 OF TUMOR GROWTH

HOURS AFTER TRITIATED THYMIDINE INJECTION	NUMBER OF MITOSIS	LABELED MITOSIS	PERCENTAGE LABELED MITOSIS *
1	200	24	12.00
2	200	126	63.00
4	200	191	95.50
6	200	190	95.00
8	200	165	82.50
10	200	136	68.00
12	200	109	54.50
14	200	105	52.50
16	201	89	44.29
18	200	121	60.50
20	200	115	57.50
22	200	106	53.00
24	200	90	45.00
26	202	97	48.02
28	200	108	54.00

* represents a mean of two animals.

TABLE 6

PROPORTION OF B16 MELANOMA TUMOR CELLS AT ANY MOMENT IN THE PHASES OF THE CELL CYCLE IN MICE NOT TREATED WITH COLCHICINE

	INTER-PHASE	PRO-PHASE	META-PHASE	ANA-PHASE	TELO-PHASE
PERCENTAGE OF PHASES	97.4	1.3	1.0	0.1	0.2
DURATION OF PHASES (hr)	-	0.67	0.51	0.05	0.11

DURATION OF MITOSIS= 1.35 hrs (range of 1.10-1.60 hrs.)

FIGURE 6

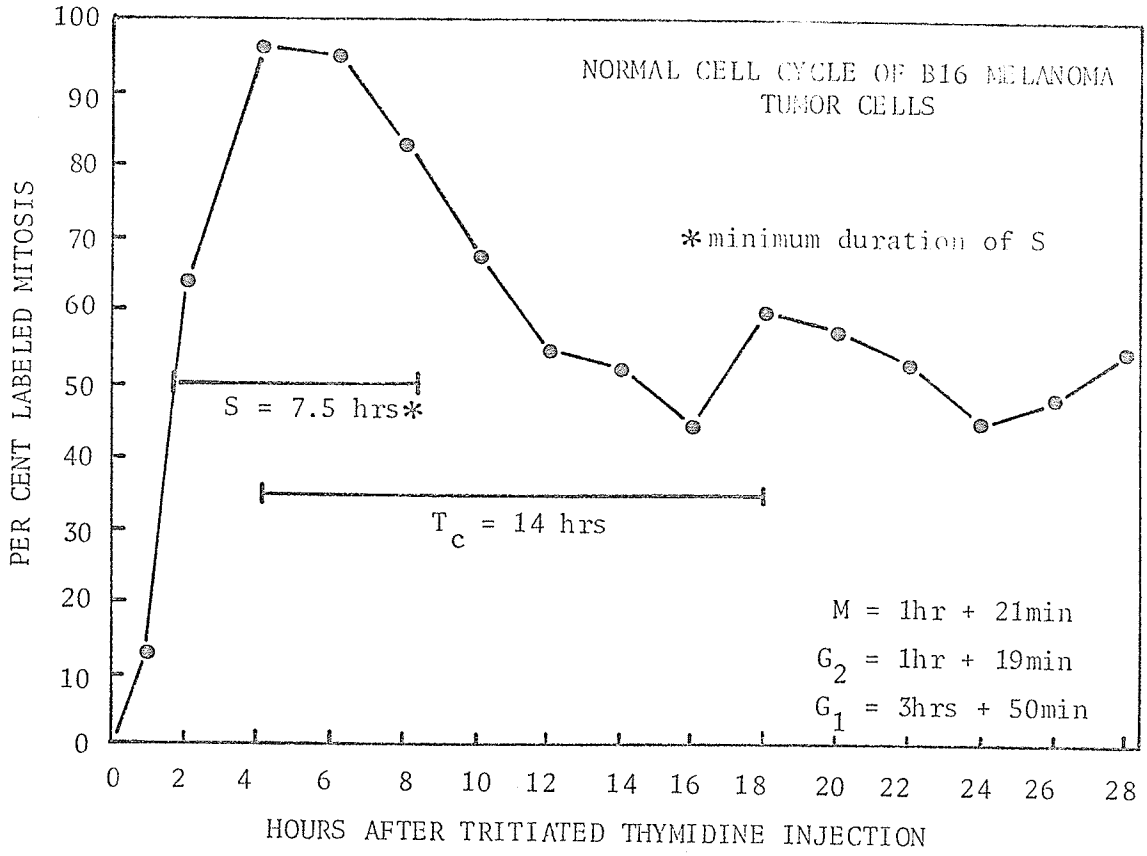


FIGURE 7

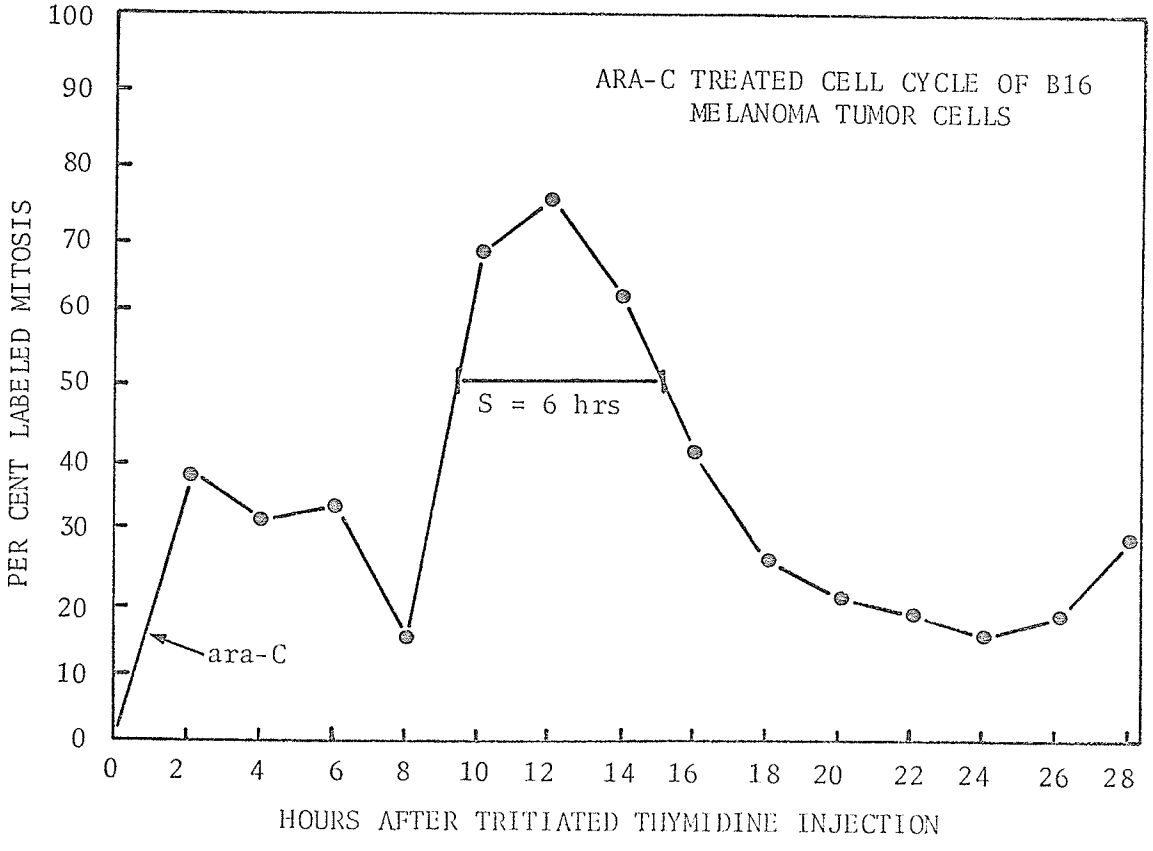


TABLE 7

CELL CYCLE OF B16 MOUSE MELANOMA ON DAY 10
AFTER THE INJECTION OF ARA-C (50mg/kg)

HOURS AFTER TRITIATED THYMIDINE INJECTION	HOURS AFTER ARA-C INJECTION	NUMBER OF MITOSIS	LABELED MITOSIS	PERCENT LABELED MITOSIS
2	1	212	78	36.38 ± 6.26
4	3	157	49	30.32 ± 2.86
6	5	143	45	31.42 ± 8.67
8	7	179	26	14.68 ± 3.60
10	9	184	128	67.76 ± 6.86
12	11	192	142	74.14 ± 4.53
14	13	255	154	61.39 ± 5.43
16	15	206	70	40.08 ±14.43
18	17	192	48	25.52 ± 2.17
20	19	277	54	20.13 ± 2.77
22	21	400	71	17.75 ± 2.56
24	23	400	61	15.25 ± 2.98
26	25	386	72	18.48 ± 3.52
28	27	400	117	29.25 ± 2.71

the effect on the mitotic activity of tumors from mice receiving four injections of ara-C (50 mg/kg), at eight hour intervals, on days 6 and 10 after tumor transplantation, with a period intervening between the two sets of ara-C administrations. The percentage of metaphases of the various groups are presented in table 8, and the observations of the experiment are graphically illustrated in Figure 8. A mean control mitotic rate was determined for the B16 melanoma, on day 10 of tumor growth, from experiment number M1. This value of 7.69 percent represents the mean mitotic rate for a four hour period. This value only acts as an indicator of how the mitotic rate is affected by the various dose schedules of ara-C as seen in Figure 8. The tumors of the first group of treated mice, 8 hours after the last ara-C injection on day 6, displayed a mitotic rate of 0.47 percent. Those of the second group, after a three day recovery period, had a mitotic rate of 7.11 percent. The mitotic rate of the tumors after four more injections declined again to a low of 0.42 percent, and following a three day rest period, the mitotic rate still remained relatively low at 1.51 percent.

One animal died in the second group of treated mice. In the fourth group, that is after the second host recovery period, three animals died, and beyond this all animals of the remaining group died. This considerable loss of animals signified that the ara-C dosages were too toxic for the mice.

Experiment No: M7

Because the dosage administered in the previous experiment appeared to be too toxic to the animals, and some degree of recovery of the mitotic

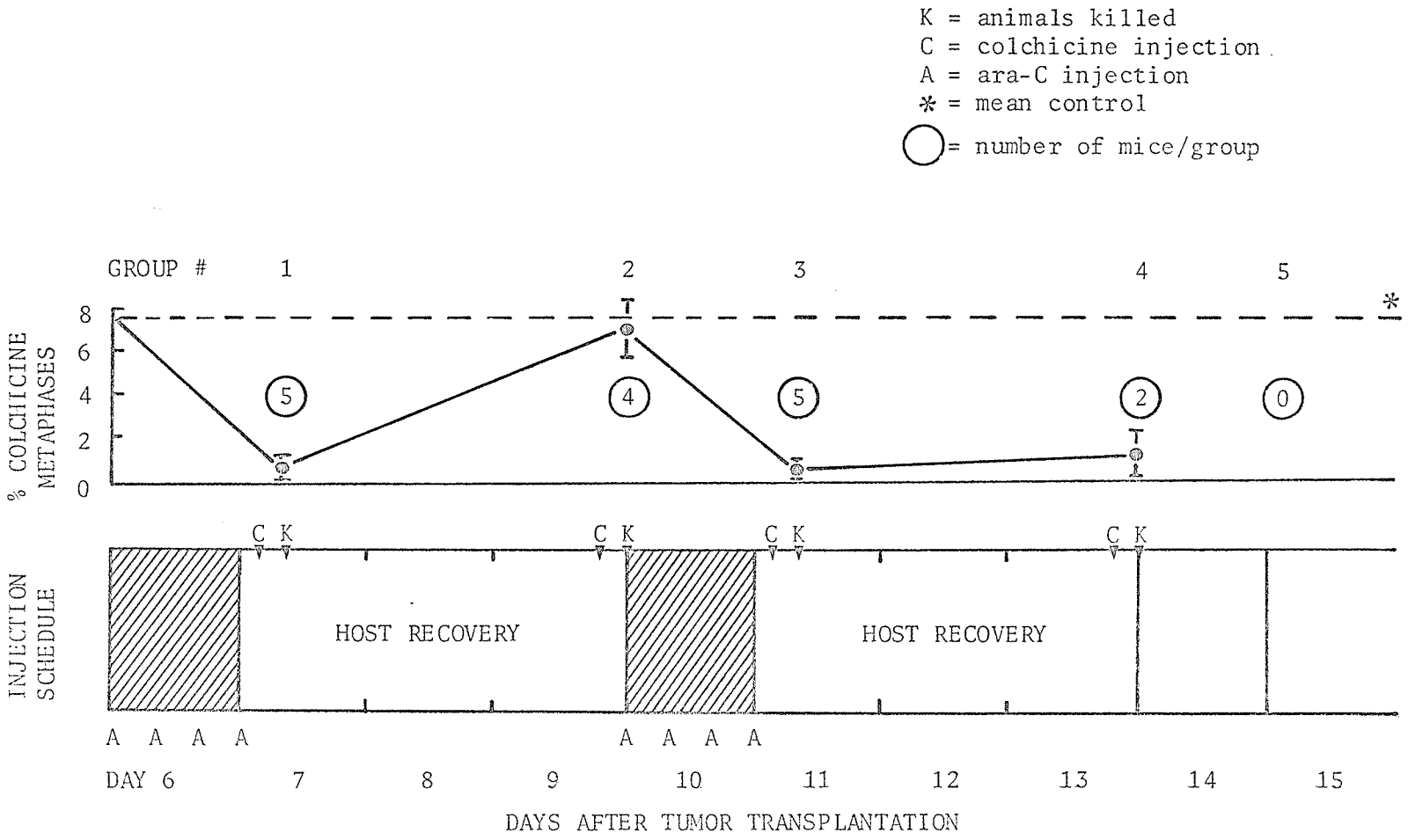
TABLE 8

MITOTIC RATES OF B16 MELANOMA AFTER FOUR INJECTIONS OF ARA-C (50mg/kg) AT EIGHT HOUR INTERVALS ON DAYS 6 AND 10

GROUP	NO. OF SURVIVORS / NO. IN GROUP	NUMBER OF NUCLEI	NUMBER OF METAPHASES	PERCENTAGE OF METAPHASES
1	5/5	20,432	97	0.47 ± 0.09
2	4/5	16,908	1,199	7.11 ± 1.38
3	5/5	21,516	94	0.42 ± 0.05
4	2/5	8,585	130	1.50 ± 0.94
5	0/5	-	-	-

FIGURE 8

MITOTIC RATES OF B16 MELANOMA AFTER FOUR INJECTIONS OF ARA-C (50mg/kg) AT EIGHT HOUR INTERVALS ON DAYS 6 AND 10



rate occurred, the dosage was reduced to half, that is 25 mg/kg and the host recovery time was reduced to a single day.

The percentage of metaphases of the various groups are listed in Table 9, and graphically illustrated in Figure 9. The mitotic rates became gradually reduced to 0.97 0.71 and 0.58 percent, respectively after each series of four injections. Moreover, there was little recovery of the mitotic rate. But the repeated dosages of ara-C still remained toxic to the animals, one mouse dying in group 5 after 10 days and all the animals of group 6 dying.

Experiment No: M8

Consequently ara-C was reduced still further to 12.5 mg/kg in this experiment, to ascertain whether this low dosage level still exerted a sufficient effect on the mitotic rate of the B16 melanoma. The host recovery period was one day also in this series. The percentage of metaphases are presented for the six 4-hour groups in Table 10, and are plotted graphically in Figure 10. All animals survived this experiment. Yet, although the mitotic rate was reduced to 1.89, 1.74 and 1.81 per cent after each successive series of injections, they returned to near normal levels, or even above normal after each recovery period.

Experiment No: A1

The purpose of this experiment was to determine the daily mitotic rate of Ehrlich ascites tumor cells after administration of a 50 mg/kg dosage of ara-C on day 6 of tumor growth.

The six 4-hour groups of both the treated and control experiments are listed in Table 11, and are plotted in Figure 11. The daily mitotic

TABLE 9

MITOTIC RATES OF B16 MELANOMA AFTER FOUR INJECTIONS OF ARA-C (25mg/kg) AT EIGHT HOUR INTERVALS ON DAYS 6, 8 AND 10

GROUP	NO. OF SURVIVORS / NO. IN GROUP	NUMBER OF NUCLEI	NUMBER OF METAPHASES	PERCENTAGE OF METAPHASES
1	4/4	16,414	160	0.97 ± 0.17
2	4/4	16,614	235	1.40 ± 0.19
3	4/4	16,477	118	0.71 ± 0.04
4	4/4	16,438	532	2.78 ± 0.31
5	3/4	12,467	72	0.57 ± 0.13
6	0/4	-	-	-

FIGURE 9

MITOTIC RATES OF B16 MELANOMA AFTER FOUR INJECTIONS OF ARA-C (25mg/kg) AT EIGHT HOUR INTERVALS ON DAYS 6, 8 AND 10

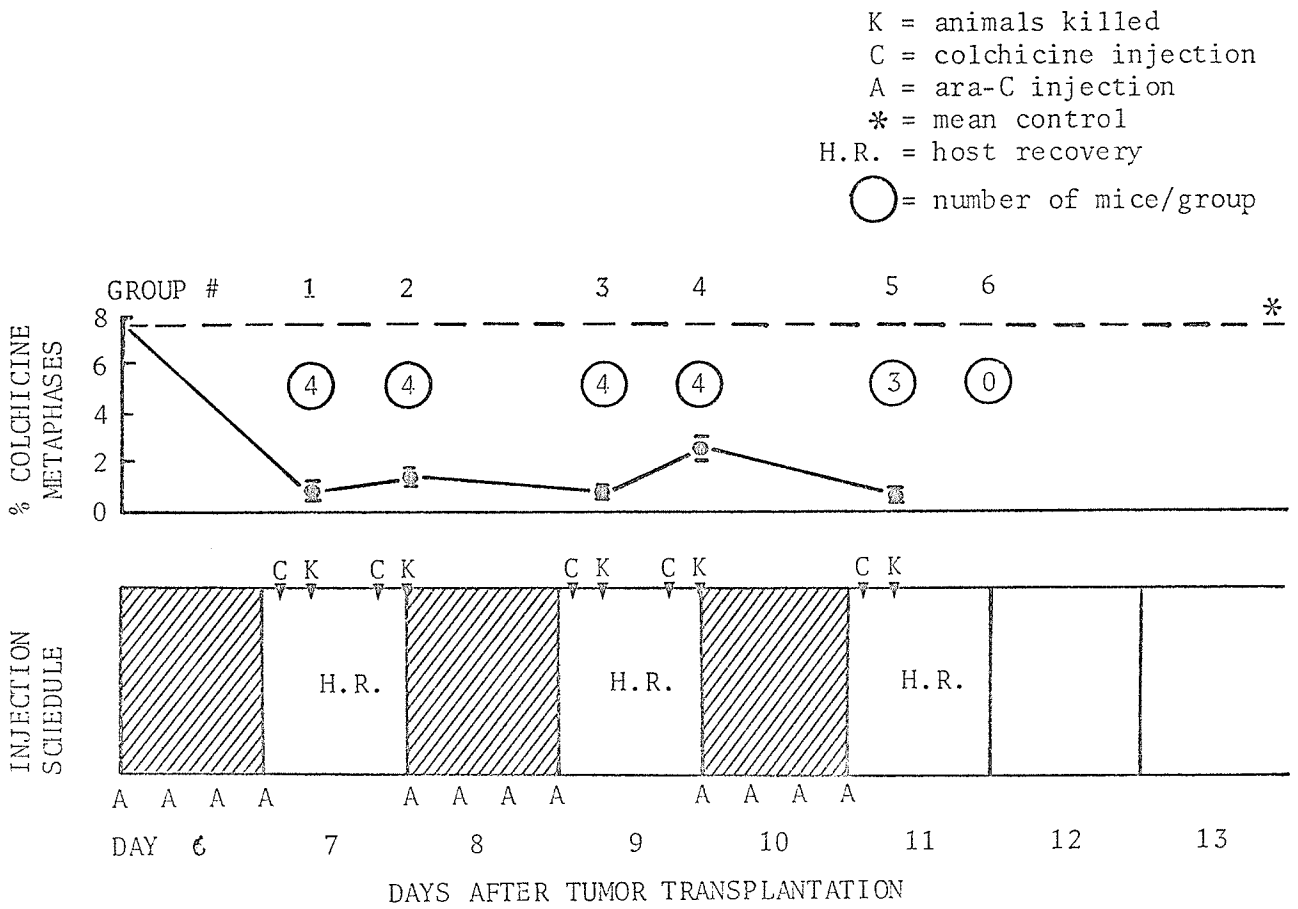


TABLE 10

MITOTIC RATES OF B16 MELANOMA AFTER FOUR INJECTIONS OF ARA-C (12.5mg/kg) AT EIGHT HOUR INTERVALS ON DAYS 6, 8 AND 10

GROUP	NO. OF SURVIVORS / NO. IN GROUP	NUMBER OF NUCLEI	NUMBER OF METAPHASES	PERCENTAGE OF METAPHASES
1	4/4	16,565	313	1.89 ± 0.37
2	4/4	16,512	1,536	9.32 ± 1.14
3	4/4	16,434	286	1.74 ± 0.51
4	4/4	16,739	1,818	10.91 ± 1.31
5	4/4	16,326	297	1.81 ± 0.66
6	4/4	16,434	853	5.12 ± 1.22

FIGURE 10

MITOTIC RATES OF B16 MELANOMA AFTER FOUR INJECTIONS OF ARA-C (12.5mg/kg) AT EIGHT HOUR INTERVALS ON DAYS 6, 8 AND 10

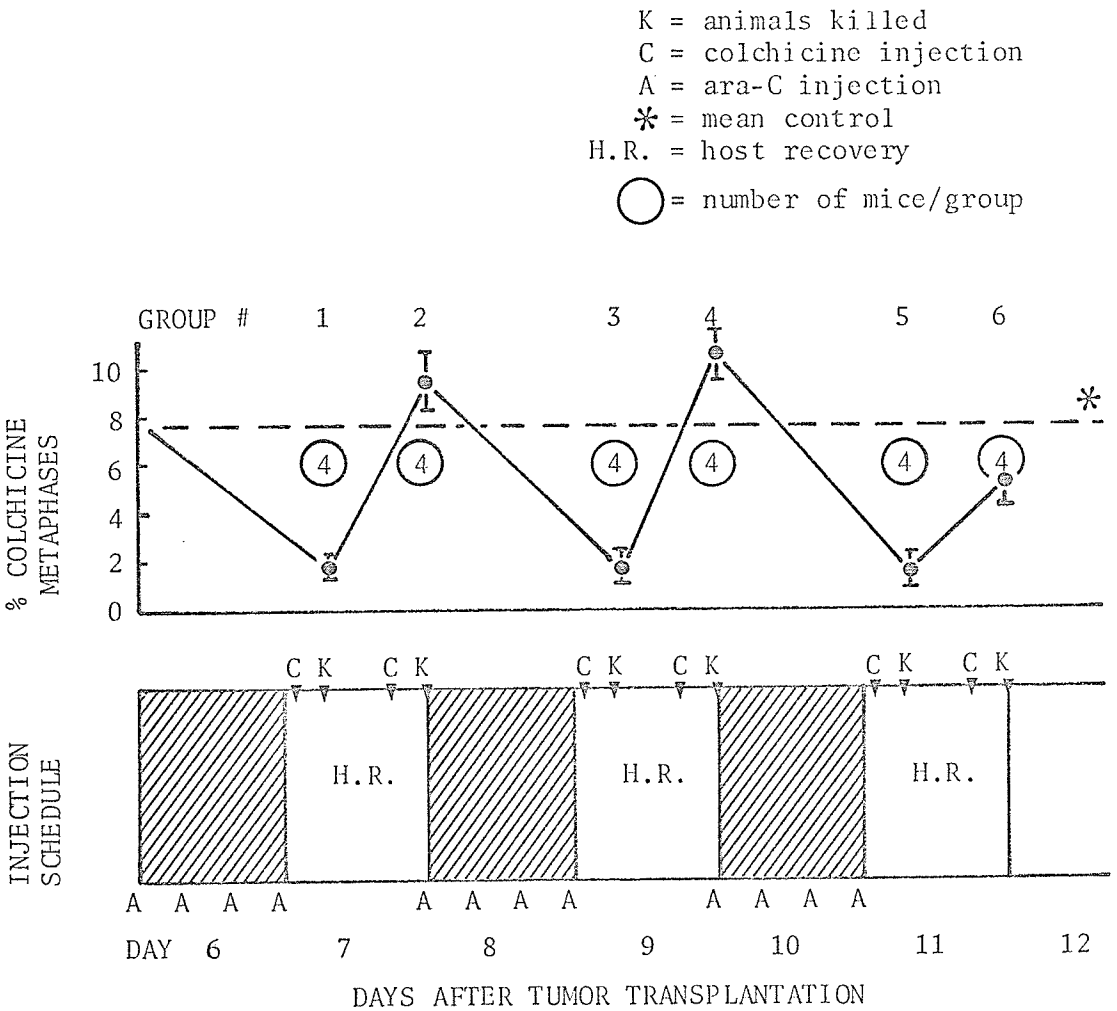


TABLE 11

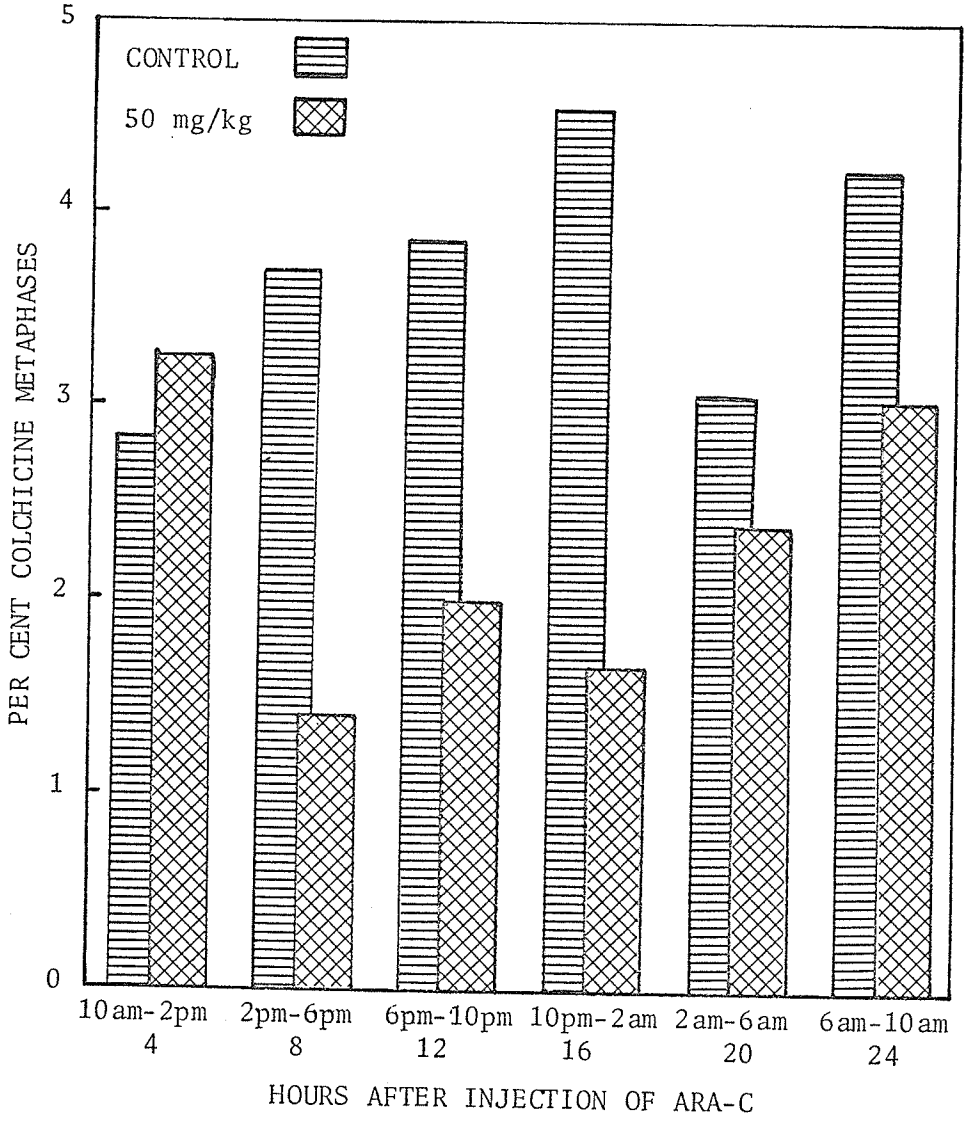
DAILY MITOTIC RATE OF EHRLICH ASCITES TUMOR CELLS ON DAY 6 AFTER A SINGLE (50mg/kg) INJECTION OF ARA-C

DOSAGE OF ARA-C (mg/kg)	TIME OF DAY	NUMBER OF NUCLEI	NUMBER OF METAPHASES	PERCENTAGE METAPHASES	DAILY MITOTIC RATE
CONTROL	10am-2pm	17,116	495	2.86 ± 0.53	
	2pm-6pm	16,701	621	3.71 ± 0.30	
	6pm-10pm	17,410	671	3.86 ± 0.53	
	10pm-2am	16,871	767	4.54 ± 0.49	
	2am-6am	16,580	511	3.07 ± 0.31	
	6am-10am	12,170*	515	4.23 ± 0.80	22.27 ± 2.96
50	10am-2pm	16,935	556	3.27 ± 0.22	
	2pm-6pm	17,245	243	1.40 ± 0.11	
	6pm-10pm	17,375	348	2.00 ± 0.23	
	10pm-2am	16,827	281	1.66 ± 0.16	
	2am-6am	16,487	397	2.40 ± 0.50	
	6am-10am	12,877*	395	3.05 ± 0.80	13.78 ± 2.02

* one animal died.

FIGURE 11

THE MITOTIC PATTERN OF 50mg/kg ARA-C TREATED AND CONTROL EHRLICH ASCITES TUMOR CELLS



rate of the control was determined, by summation of the six 4-hour percentages, to be 22.27 percent. Although considerable variation was observed during the 24 hour period no significant difference was found between the six groups, see Table 11. The daily mitotic rate represented the number of cells added to the total tumor cell population every 24 hours. The doubling time for the Ehrlich ascites tumor cells was thus 107 hours or 4.4 days.

It is important to emphasize at this point, that this strain of Ehrlich ascites tumor cells proliferated at a much slower rate, merely at about one quarter of the rate observed for the same strain by previous authors (Bertalanffy et al., 1965). To ascertain whether a genetic modification may have occurred since the tumor was used last, a chromosomal analysis was performed. This signified that the modal chromosome number of the ascites tumor cells had changed from 74 in 1963-1965 when the tumor was used by Bertalanffy, to 71 at present. This genetic change may account for the considerable deceleration of the growth rate of the tumor, as observed in the present series.

In the groups of mice treated with ara-C the mitotic rate of the Ehrlich ascites tumor varied significantly. Yet it was interesting to note that the group killed four hours after an injection of ara-C remained statistically identical to the control value of the untreated tumor ($p > 0.01$). Eight hours after an injection of ara-C the mitotic rate declined to 1.40 percent from the 3.71 control level. The mitotic rates of tumors from animals 12, 16, 20 and 24 hours after the injection of ara-C exhibited

a slow recovery with that of the 24-hour group approaching the control level.

Experiment No: A2.

The purpose of this experiment was to determine the effect of two and three serial injections of ara-C, given at daily intervals, upon the mitotic rate of Ehrlich ascites tumor cells.

The percentage of metaphases arrested by colchicine during the four hour period in the tumors of the two and three injection groups, and with their control values, are shown in Table 12. The tumors receiving two injections of ara-C displayed a mitotic rate of 1.81 percent, which was significantly different from that of the mitotic rate of 3.29 in the control group. The tumors of the group receiving three injections of ara-C exhibited a mitotic rate of 1.65, which was significantly different from that of the control group with a mitotic rate of 3.05 percent. Yet, there was no significant difference between the mitotic rate of the two treated groups of tumors ($P > 0.05$).

Experiment No: A3

The aim of this experiment was to determine whether pretreatment with ara-C exerted any effect upon the transplantability of Ehrlich ascites tumor cells. A group of mice received transplanted ascites effusion from a donor mouse injected with a single dose of ara-C, four hours before transplanting. The EAT cells were allowed to grow for six days then received an injection of colchicine at 10:00 a.m. and were killed at 2:00 p.m. The mitotic rate of the tumor was 2.00 percent (Table 13). The

TABLE 12

EFFECT OF 2 AND 3 INJECTIONS OF ARA-C (50mg/kg) ON THE MITOTIC RATE OF EHRLICH ASCITES TUMOR CELLS

NUMBER OF INJECTIONS	ON DAYS	NUMBER OF NUCLEI	NUMBER OF METAPHASES	PERCENTAGE OF METAPHASES
2	6,7	16,877	310	1.81 ± 0.22
CONTROL		16,570	546	3.29 ± 0.53
3	6,7,8	16,994	283	1.65 ± 0.14
CONTROL		16,675	510	3.05 ± 0.19

TABLE 13

TRANSPLANTABILITY OF EHRLICH ASCITES TUMOR CELLS AFTER 1, 2 AND 3 INJECTIONS OF ARA-C (50mg/kg)

NUMBER OF INJECTIONS	NUMBER OF NUCLEI	NUMBER OF METAPHASES	PERCENTAGE OF METAPHASES
1	16,714	335	2.00 ± 0.24
2	16,475	463	2.80 ± 0.29
3	16,894	462	2.75 ± 0.35

Ehrlich tumors of mice receiving transplanted ascitic effusions from donor mice pretreated with two and three dosages of ara-C exhibited mitotic rates of 2.80 and 2.75 percent respectively after a week of tumor growth. The mitotic rates did not differ significantly from the control values of experiment A1 ($P > 0.05$).

Experiment No: A4

The purpose of this experiment was to ascertain the effect of a single injection of ara-C on the thymidine index of Ehrlich ascites tumor cells. Groups of mice were killed at various time intervals after being treated with ara-C and having received an intraperitoneal injection of tritiated thymidine 15 minutes prior to sacrifice. The thymidine index was determined in the Ehrlich ascites tumor cells of four untreated control groups of mice at ½, 1, 12 and 24 hour intervals after the beginning of the experiment. These thymidine indices of tumors from both treated and control groups are presented in Table 14, and plotted against time in Figure 12. It is evident that the thymidine index declined from the control value to 4.10 percent by 15 minutes in the ara-C treated tumors. The thymidine index reached a low of 0.19 percent 30 minutes after the injection of ara-C, and by 45 minutes recovery had begun. Recovery was gradual, attaining the level of the control values 22 hours after ara-C administration. However, 24 hours after treatment, the thymidine index was 30.49 percent, that is, considerably higher than the control level.

Experiment No: A5

The purpose of this experiment was to determine the cell cycle times

TABLE 14

EFFECT OF A SINGLE INJECTION OF ARA-C (50mg/kg) UPON THE THYMIDINE INDEX OF EHRLICH ASCITES TUMOR CELLS ON DAY 6

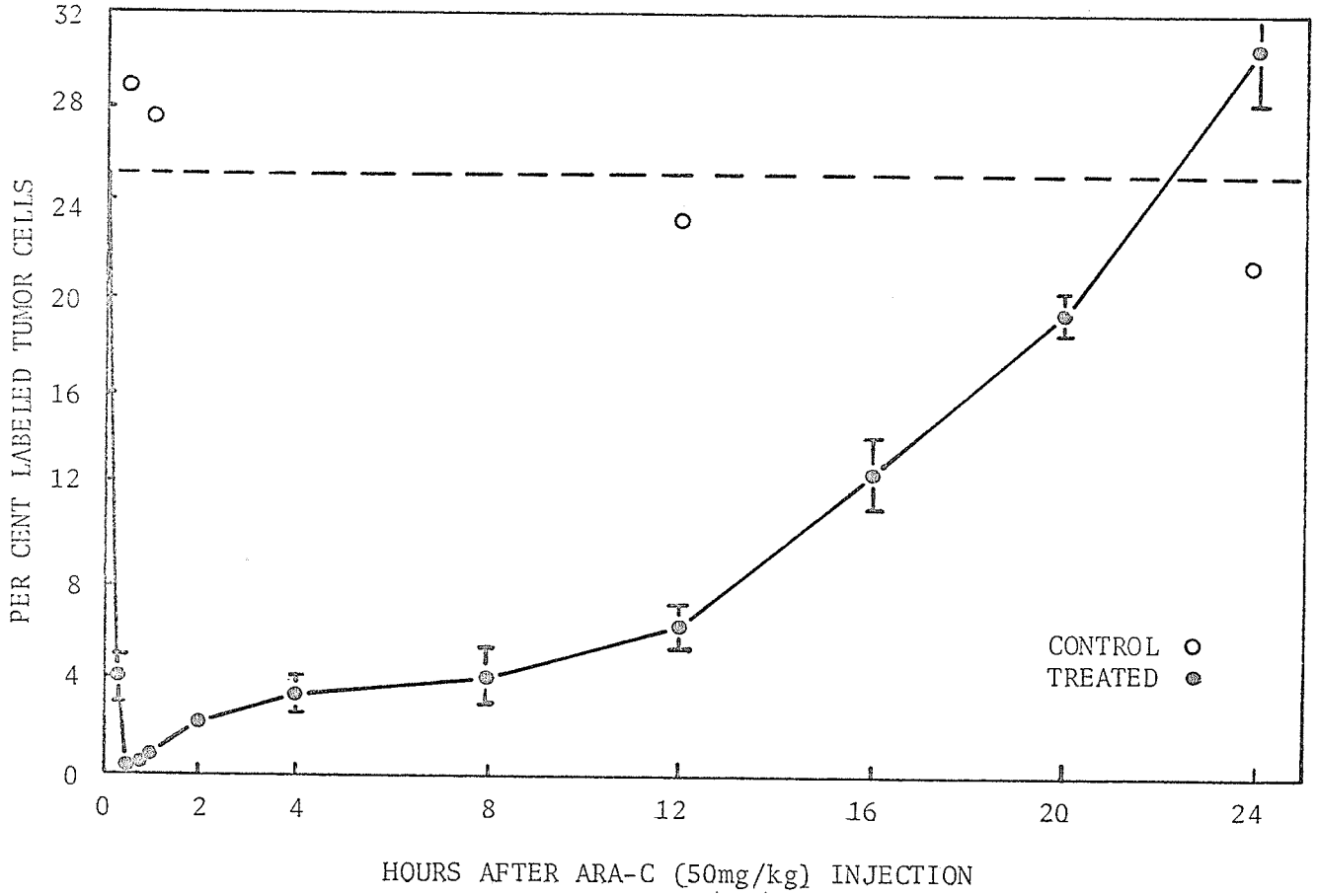
HOURS AFTER INJECTION OF ARA-C	TOTAL NUMBER OF CELLS	NUMBER OF LABELED CELLS	THYMIDINE INDEX (%)
0.25	4,029	165	4.10 ± 1.05
0.50	4,032	8	0.19 ± 0.13
(0.50)	(2,004)	(580)	(28.93)
0.75	3,026*	24	0.78 ± 0.51
1	3,136*	26	0.82 ± 0.10
(1)	(2,031)	(555)	(27.31)
2	4,243	91	2.13 ± 0.27
4	4,244	143	3.34 ± 0.28
8	4,121	167	4.07 ± 1.21
12	4,344	275	6.34 ± 1.06
(12)	(2,118)	(490)	(23.18)
16	4,356	565	12.90 ± 1.70
20	4,615	898	19.40 ± 0.73
24	4,206	1,278	30.49 ± 2.05
(24)	(2,121)	(456)	(21.50)

* one mouse died.

( )control groups with two mice each.

FIGURE 12

THYMIDINE INDEX OF EHRLICH ASCITES TUMOR CELLS AT VARIOUS INTERVALS AFTER THE INJECTION OF ARA-C



of normal and ara-C treated Ehrlich ascites tumor cells. The percentage of labeled mitoses of the untreated tumor (Table 15) were plotted against time after tritiated thymidine injection (Figure 13). From these data, the total cell cycle time (t_C) was estimated to be 16 hours, with a S phase duration of 10 hours. The period of the G_2 phase plus one half the duration of mitosis was calculated to be 3-3/4 hours. The duration of mitosis of Ehrlich ascites tumor cells was determined in tumor bearing mice (not treated with colchicine) to be two hours and 17 minutes (Table 16). Consequently the length of the G_2 phase equaled $2\frac{1}{2}$ hours, according to the calculation: 3-3/4 hours - $\frac{2\frac{1}{4}}{2}$ = $2\frac{1}{2}$ hours (approximately) and the G_1 phase equals $t_C - (G_2 + M + S)$ or, 16 hours - $(2\frac{1}{2} + 2\frac{1}{4} + 10)$ = $1\frac{1}{4}$ hours.

The percentage of labeled mitoses of the tumors of ara-C treated groups of mice (Table 17) were plotted against time after the injection of a single dose of tritiated thymidine (Figure 14). Ara-C was administered one hour after the injection of tritiated thymidine. The first curve of the plot expressing the cell cycle, was affected rather drastically, reaching a peak of only 21 percent, and then falling to zero only seven hours after ara-C administration. Two hours later the percentage labeled mitoses had recovered to 8.87 percent, and 17 hours after ara-C treatment attained 80 percent, representing the peak of the delayed first mitotic wave. The rise of percentage mitotic figures ceased at this point forming a plateau for 6 hours, and this was followed by a sharp decline 25 hours after the injection of ara-C. The S-phase of

TABLE 15

CELL CYCLE OF UNTREATED EHRLICH ASCITES TUMOR CELLS ON DAY 6 OF TUMOR GROWTH

HOURS AFTER TRITIATED THYMIDINE INJECTION	NUMBER OF MITOSIS	LABELED MITOSIS	PERCENTAGE LABELED MITOSIS*
1	203	44	21.66
2	200	50	25.00
4	200	103	51.50
6	201	180	89.55
8	200	173	86.50
10	202	163	80.69
12	200	169	84.50
14	200	95	47.50
16	202	121	58.96
18	201	134	66.63
20	202	142	70.29
22	200	150	75.00
24	202	148	73.30
26	200	128	64.00
28	204	114	55.76

* represents a mean of two animals.

TABLE 16

PROPORTION OF EHRLICH ASCITES TUMOR CELLS AT ANY MOMENT IN THE PHASES OF THE CELL CYCLE IN MICE NOT TREATED WITH COLCHICINE

	INTER-PHASE	PRO-PHASE	META-PHASE	ANA-PHASE	TELO-PHASE
PERCENTAGE OF PHASES	96.9	1.1	0.2	0.2	1.4
DURATION OF PHASES (min)	-	55.6	10.5	7.9	63.2

DURATION OF MITOSIS= 2.28 hrs (range of 2.00-3.28 hrs.)

FIGURE 13

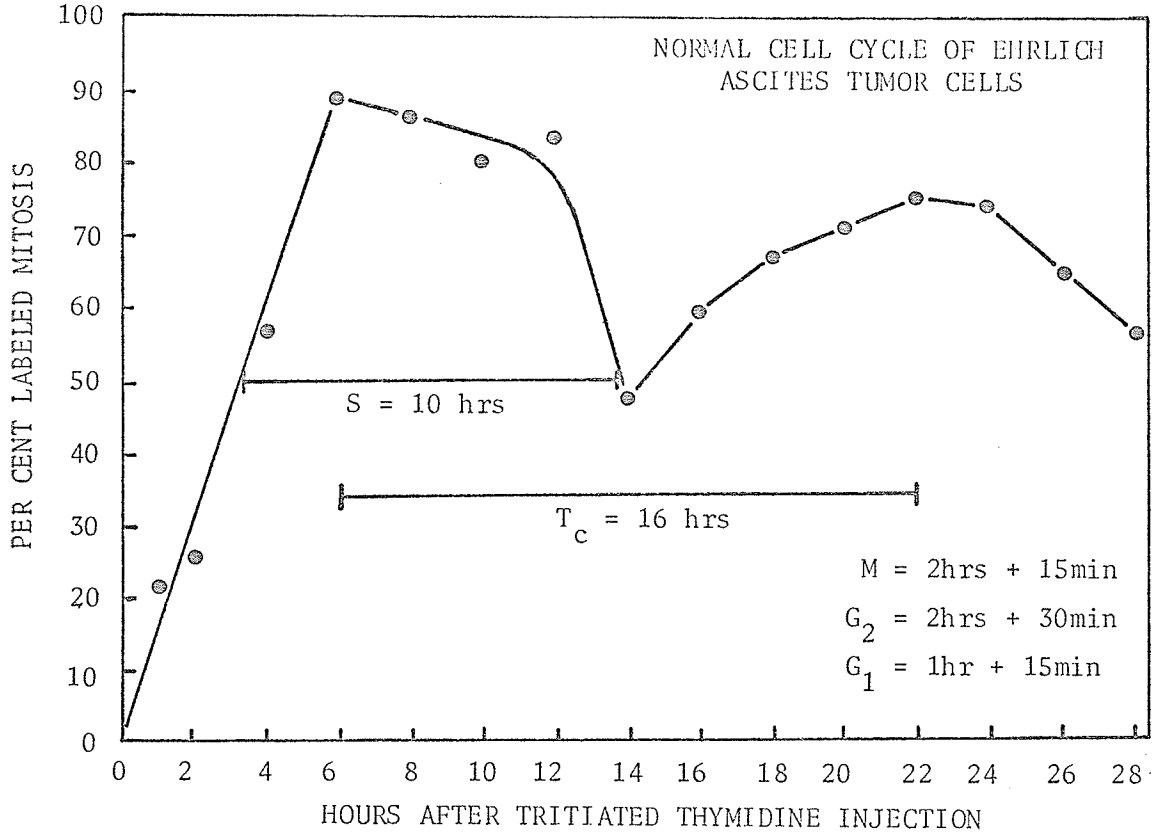


FIGURE 14

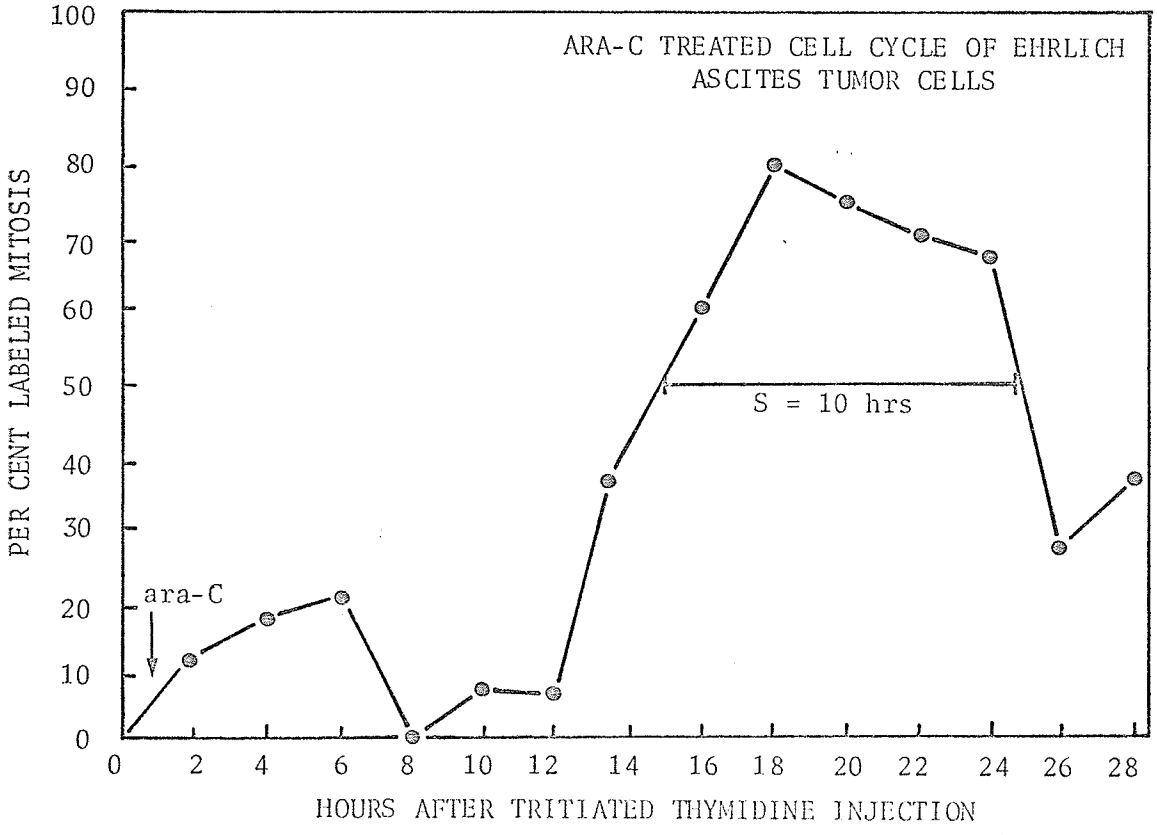


TABLE 17

CELL CYCLE OF EHRLICH ASCITES TUMOR CELLS ON DAY 6 AFTER THE INJECTION OF ARA-C (50mg/kg)

HOURS AFTER TRITIATED THYMIDINE INJECTION	HOURS AFTER ARA-C INJECTION	NUMBER OF MITOSIS	LABELED MITOSIS	PERCENT LABELED MITOSIS
2	1	360	40	11.20 ± 1.44
4	3	148	24	17.04 ± 3.31
6	5	61	13	21.05 ± 1.23
8	7	3	0	0
10	9	62	7	8.87 ± 3.44
12	11	27	4	8.75 ± 5.90
14	13	61	23	36.93 ± 2.91
16	15	159	100	60.03 ± 5.93
18	17	200	160	80.25 ± 1.75
20	19	329	250	74.12 ± 3.79
22	21	308	219	71.59 ± 4.02
24	23	303	203	66.75 ± 1.10
26	25	179	47	28.40 ± 4.32
28	27	221	85	36.41 ± 6.64

the cell cycle of treated Ehrlich ascites tumor cells was estimated to be approximately 10 hours. Indications of a second wave are apparent in Figure 14, but this was beyond the range of the present experiment.

Experiment No: A6

The purpose of this experiment was to study the effect on Ehrlich ascites tumor cells of four injections of ara-C (25 mg/kg), administered at eight hour intervals, on days 3, 5 and 7 after transplantation, allowing one day rest periods on days 4, 6 and 8. The percentage of metaphases of tumors from the different animal groups thus treated are presented in Table 18. The data are also plotted graphically in Figure 15. A mean control mitotic rate for the EAT cells, on day 6 of tumor growth was determined in experiment number A1. This value of 3.71 percent represents the mean mitotic rate for a four hour period and was only used as an indicator of how the mitotic rate was affected by the various dose schedules of ara-C as seen in Figure 15. The mitotic rates of the tumors were reduced to 1.75, 0.44 and 0.79 percent, respectively, after each one of the series of four injections. A recovery of the mitotic rate did not occur after the first host rest period, although a slight rise was apparent in the tumor groups following both the second and third host rest periods. One animal in the sixth group died, and the mice of the entire seventh group succumbed to the effect of ara-C treatment.

TABLE 18

MITOTIC RATES OF EHRLICH ASCITES TUMOR CELLS AFTER FOUR INJECTIONS OF ARA-C (25mg/kg) AT EIGHT HOUR INTERVALS ON DAYS 3, 5 AND 7

GROUP	NO. OF SURVIVORS / NO. IN GROUP	NUMBER OF NUCLEI	NUMBER OF METAPHASES	PERCENTAGE OF METAPHASES
1	4/4	16,394	287	1.75 ± 0.89
2	4/4	16,408	272	1.65 ± 0.21
3	4/4	16,568	74	0.44 ± 0.05
4	4/4	16,655	254	1.51 ± 0.22
5	4/4	16,368	132	0.79 ± 0.30
6	3/4	12,577	117	0.92 ± 0.10
7	0/4	-	-	-

FIGURE 15

MITOTIC RATES OF EHRLICH ASCITES TUMOR CELLS AFTER FOUR INJECTIONS OF ARA-C (25mg/kg) AT EIGHT HOUR INTERVALS ON DAYS 3, 5 AND 7

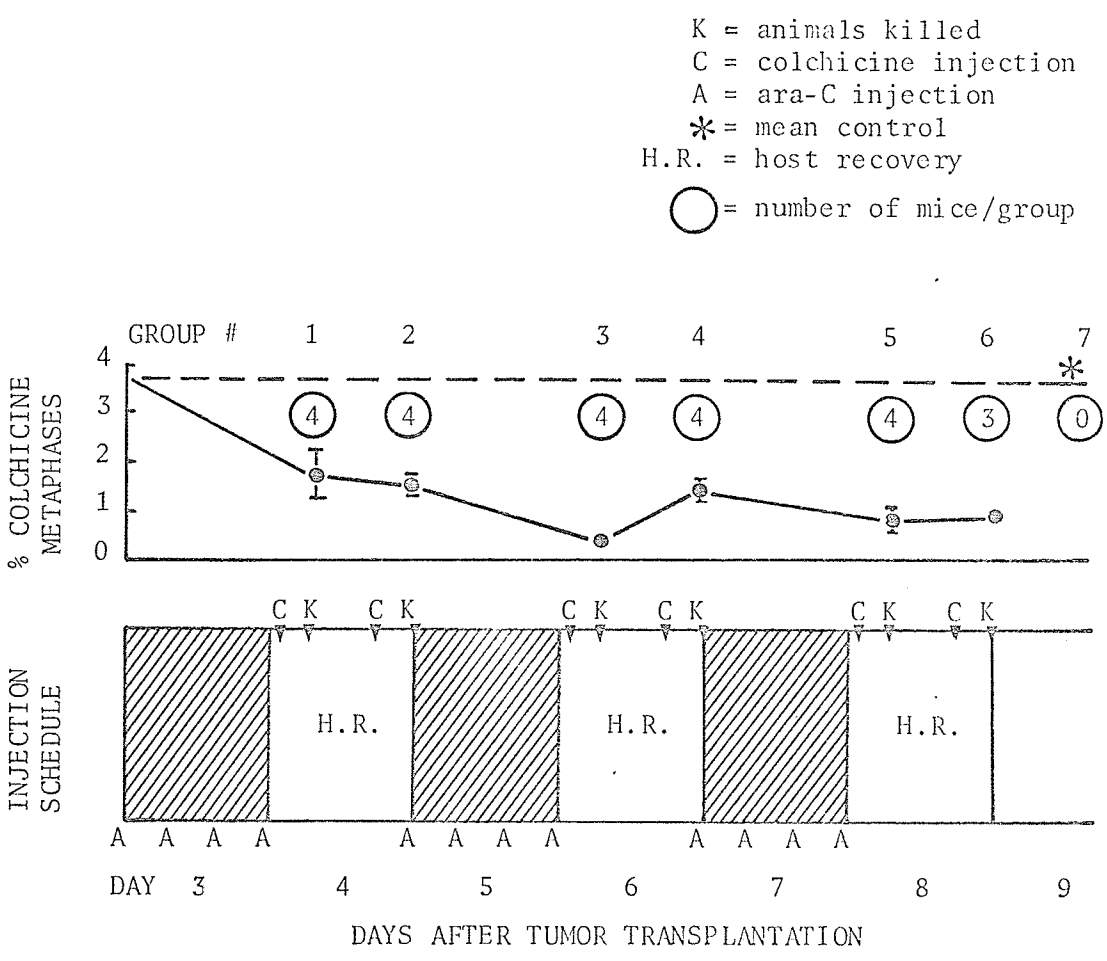


Photo 1. Mouse duodenum, 4 hours after receiving a total of 5 mg. of ara-C over a 24 hour period. Note the karyorrhexis of the epithelial cells of the crypts of Lieberkühn and the lining of the villi. H & E x64.

Photo 2. Mouse duodenum, 4 hours after receiving a total of 10 mg. of ara-C over a 48 hour period. Note the reduction in the length of the villi as compared to photo 1. H & E x64.

Photo 3. Mouse duodenum, 4 hours after receiving a total of 15 mg. of ara-C over a 72 hour period. Note the complete absence of villi and crypts, while the remaining epithelial cells are being shed. H & E x64.

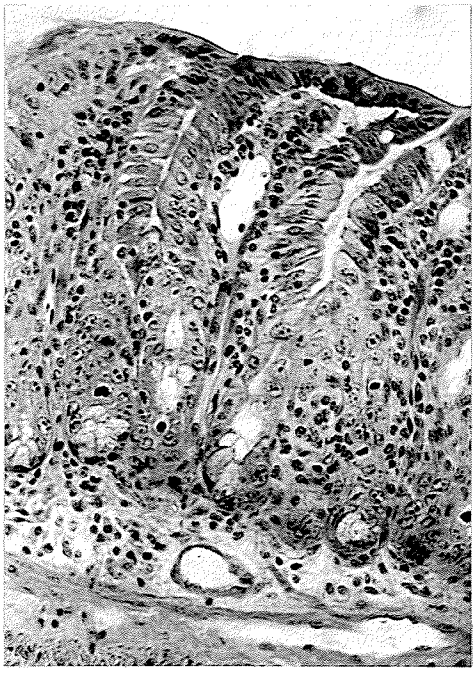


Photo 1

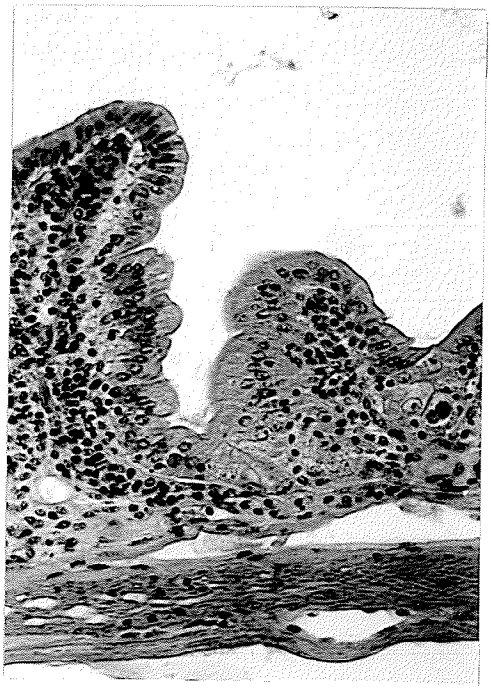


Photo 2

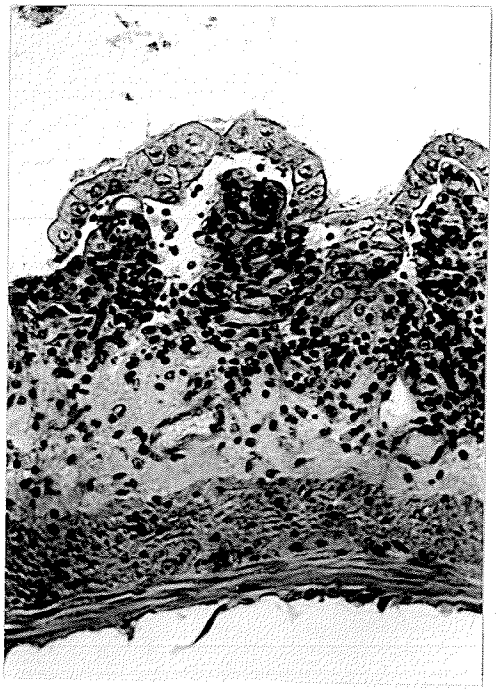


Photo 3

Photo 4. A high powered view of a crypt of Lieberkühn showing the accumulation of the nucleoli in some cells into one large mass and the over all distension of the crypt cells after 10 mg. of ara-C injected over a 48 hour period. H & E x256.

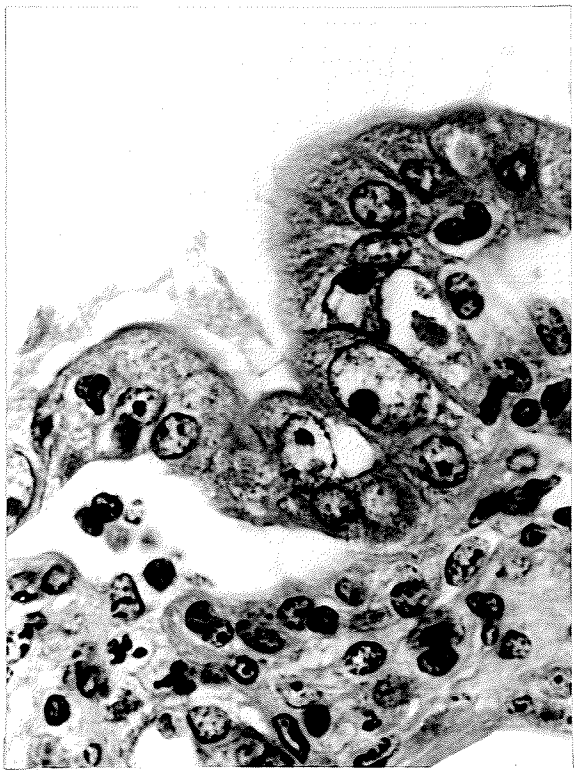


Photo 4

Photo 5. B16 mouse melanoma, four hours after the injections of colchicine. Note the typical colchicine metaphases. H & E x160

Photo 6. B16 mouse melanoma, 4 hours after the second sequence of four ara-C injections (50 mg/kg) with a 3-day host recovery period between each sequence. Note the general increase in size and the melanin granules in the cytoplasm of some cells. H & E x160

Photo 7. High powered view of B16 mouse melanoma after the same treatment as in photo 6. Again note the high concentration of melanin granules in the cytoplasm of some cells. H & E x256

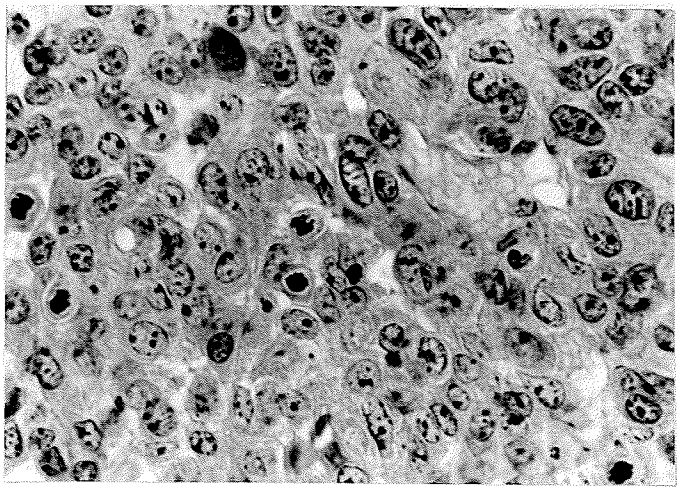


Photo 5

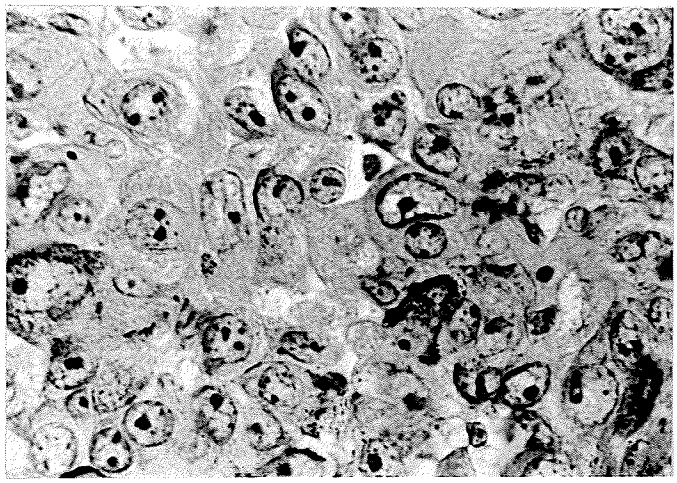


Photo 6

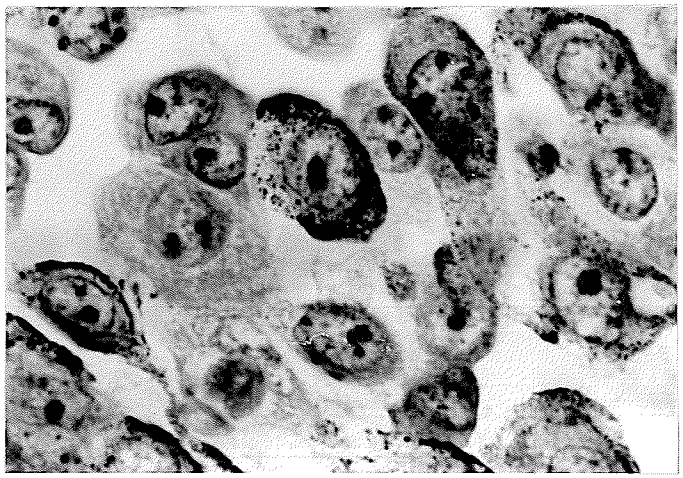


Photo 7

Photo 8. EAT cells, four hours after the injection of colchicine. Note the colchicine metaphases. H & E x160.

Photo 9. EAT cells, 4 hours after the second sequence of four ara-C injections (25 mg/kg) with a 1-day host recovery period between each sequence. Note the large nucleoli and the general increase in size over the untreated cells in photo 8. H & E x160.

Photo 10. High powered view of the EAT cells after the same treatment as in photo 9. Again note the large nucleoli. H & E x256.

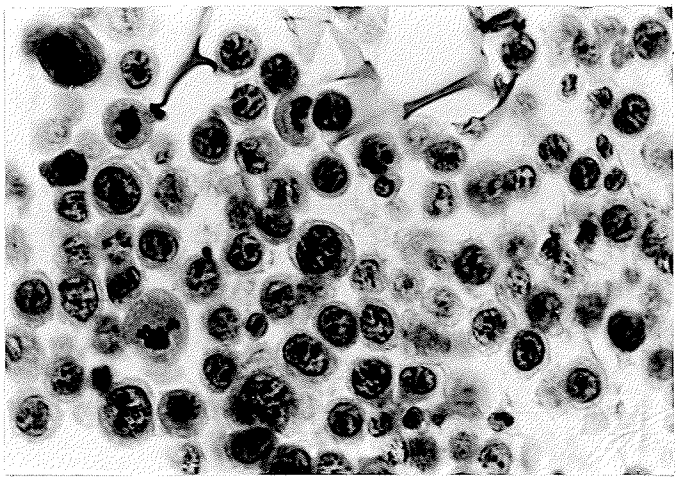


Photo 8

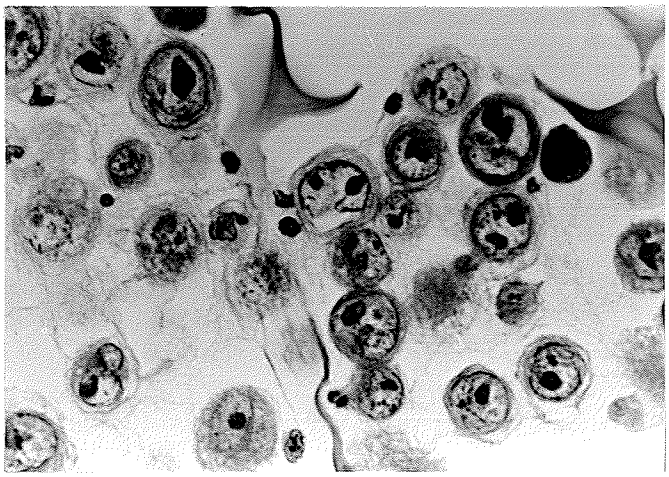


Photo 9

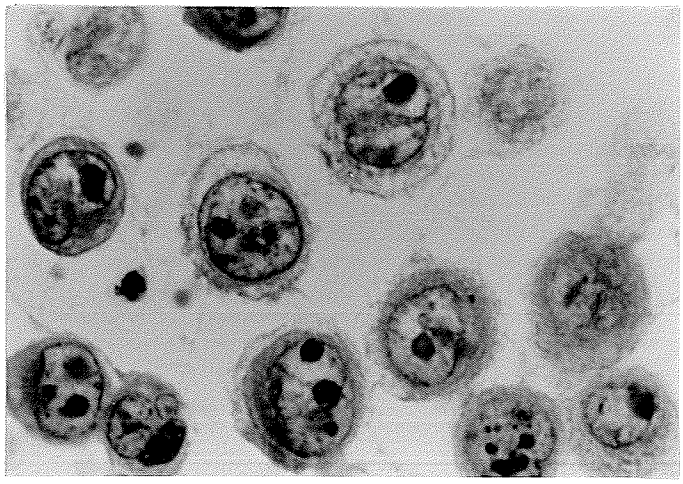


Photo 10

Photo 11. Autoradiograph of the B16 mouse melanoma after an injection of tritiated thymidine. Note the labeled metaphase. H & E x256.

Photo 12. Autoradiograph of EAT cells after an injection of tritiated thymidine. Note the labeled metaphase in the center of the field. H & E x256.

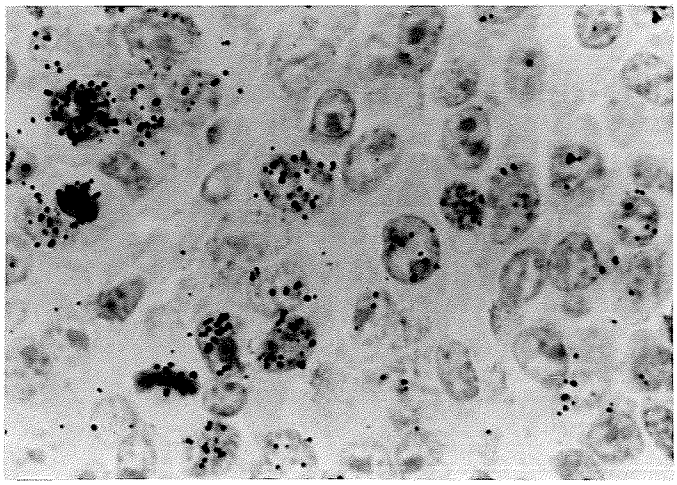


Photo 11

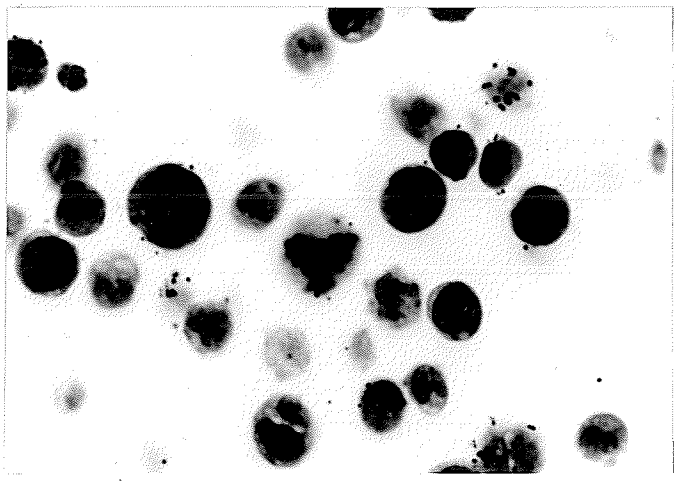


Photo 12

CHAPTER V.

DISCUSSION OF RESULTS

It was found that a 50 mg/kg dose of ara-C was more effective than a 80 mg/kg dose, in retarding the B16 melanoma. Three successive daily injections of ara-C were most effective against both the B16 melanoma and the EAT cells, while ara-C had no effect against the transplantability of either of the tumors. The uptake of tritiated thymidine by tumor cells was blocked within 15 minutes of ara-C injection. The cell cycle of both the B16 melanoma and the EAT cells was delayed by ara-C and the S-phase may even have been shortened in the B16 melanoma. For multiple injections of ara-C, the 25 mg/kg was found to be the most effective and a host recovery time of one day between such treatments was found to allow the least tumor cell recovery.

The observations described indicated that in the untreated B16 melanoma about 46 percent of its total cell population divided during a 24-hour period. These observations substantiate the previous work on the B16 melanoma by Bertalanffy and McAskill (1964) who ascertained the daily mitotic rate to be 36 percent. The discrepency in the daily mitotic rate may be attributable to the circumstance that in the present work, groups of animals were sampled every four hours rather than six as in the previous study. Both studies, however, were carried out after 10 days of tumor growth. The effects of a single injection of ara-C upon the daily mitotic rate of the B16 melanoma tumor was not surprising in view of the abrupt reduction of mitosis observed by Kim and Edinoff (1965) in similarly treated Hela S-3 cells. In the present study employing both 50 and 80 mg/kg dosages of ara-C, the mitotic rate was reduced by 75 percent four hours after the injection of ara-C. The 50

mg/kg dosage of ara-C reduced the mitotic rate to 0.56 percent eight hours after administration while the 80 mg/kg dosage of ara-C depressed the mitotic rate to 0.69 percent 12 hours later. It was thus evident that the 50 mg/kg dosage of ara-C had a more profound influence upon the mitotic activity of the B16 melanoma tumor cells than did the 80 mg/kg dosage. From the low levels produced by ara-C, the mitotic rates of the tumor progressively increase because ara-C was apparently no longer present in effective levels in the blood stream. It was shown previously that ara-C, at the 50 mg/kg dosage level, did not remain effective in the blood stream of mice longer than one hour (Skipper et al., 1967). Mulligan and Mellitt (1968) observed that the half-life of ara-C in mouse serum was only 37 minutes, because ara-C was quickly deaminated to inactive ara-uracil (Camiener and Smith, 1965; and Saslow et al., 1966). The recovery of the mitotic rate was much more rapid in the tumors of animals receiving the 80 mg/kg dosage of ara-C, while the tumors of mice receiving 50 mg/kg displayed a more gradual recovery of the mitotic rate. This may be explained in part by the work of Mulligan and Mellitt (1968), and Borsa et al. (1969) who discovered that a 250 mg/kg dosage had a shorter half-life in the serum of mice than did a 50 mg/kg dosage. They could not explain why a larger dosage showed a shorter half-life but Kessel and Hall (1967) believed that such effects were attributable to the interplay of such factors as absorption, enzyme binding and dehydration. In both groups receiving ara-C the recovery of the mitotic rate of the B16 melanoma was incomplete, yet the recovery that did occur

was attained within a 24-hour period.

In a general way, similar effects were observed in the Ehrlich ascites tumor cells. The daily mitotic rate of this tumor was decreased by 38 percent after a 50 mg/kg injection of ara-C. However, no reduction of the mitotic activity was apparent until eight hours after the injection of ara-C, at which time, the mitotic rate was 1.40 percent. The reason for such a delay is yet unexplainable. Beginning 12 hours after the injection of ara-C, the mitotic rate of the EAT cells began to recover in a similar fashion to that of the B16 melanoma tumor cells.

It is imperative at this point to discuss the abnormal growth pattern of the Ehrlich ascites tumor cells in the presentation. Bertalanffy, et al. (1965), working with the same Ehrlich ascites strain some years previously, when it was characterized by 74 chromosomes and one biarmed marker chromosome, found the doubling time of the tumor cells to be about 21 hours. The EAT cells used in the present experiments were thought to be the same tumor population; however, its doubling time was found to be 107 hours. This represented a doubling time five times that of the tumor used four years ago. A chromosomal analysis of the EAT cells in the present experiments revealed that the modal chromosome number had apparently shifted since then to 71, but still retained the one biarmed marker chromosome. This change in the chromosomal number and conceivable associated alteration in genetic character, may partly explain the slower growth rate of the EAT cells in the present study. Since the experiments of Bertalanffy et al. (1965) were carried out, this strain of Ehrlich ascites cells have been maintained in female mice.

However, prior to their use in these present experiments, the EAT cells were maintained for several generations in male mice of the Connaught strain. Relevant to this phenomenon may be the observation of Vincent and Nicholls (1967), studying the growth rates of Ehrlich ascites cells in males, who found that the chromosome modal numbers of the tumor cells changed more often when they were grown in male rather than female animals. They observed that significantly less growth occurred in male mice when the tumor cells were transferred from donors of either sex. It is conceivable that if the chromosomal number altered in a sufficient number of EAT cells, this could result in a change of the overall growth pattern of the tumor also.

In the ara-C series, it was observed that three successive daily injections of the drug ara-C reduced the mitotic rate to a lower level, in both B16 melanoma and EAT cells, than did a single or two successive administrations. However, in both B16 melanoma and EAT cells those groups treated with either two or three injections of ara-C had significantly lower mitotic rates than their control groups. These observations signified that successive daily injections of ara-C exerted a greater inhibitory effect upon the mitotic rate of B16 melanoma and Ehrlich ascites tumor cells than a single injection.

Ara-C apparently affected little or not at all the transplantability of either the B16 melanoma tumor cells or the EAT cells. However, the mitotic rate was lower in the tumors in the melanoma group whose donor animal was pretreated with two injections of ara-C. The

rate of that group was statistically different from the group receiving one and three injections and the control value found in experiment M1. At present the reason for this lower mitotic rate can not be explained.

One of the most significant findings in the present series of experiments was that a single injection of ara-C affected the thymidine index in both tumor populations as soon as 15 minutes after administration of the drug, or even earlier. This signified that ara-C prevented the uptake of tritiated thymidine by cells undergoing DNA synthesis almost immediately upon administration. In both the B16 melanoma and Ehrlich ascites cells, the thymidine index declined by 84 percent within the first 15 minutes after injection of 50 mg/kg of ara-C. The minimum level of the thymidine index was attained by 45 and 60 minutes after the injection of ara-C in Ehrlich ascites and B16 melanoma cells, respectively. From approximately one hour on after the injection of ara-C, the thymidine index began to recover. This may be again attributable to the diminuation of the effective levels of ara-C in the blood one hour after the drug administration, (Skipper et al., 1967). The thymidine index recovered much faster in the B16 melanoma than in the Ehrlich ascites tumor cells. Similarly, a more rapid recovery was observed in the mitotic rates of the B16 melanoma tumor cells as compared to the recovery of those rates of the EAT cells. This rapid recovery in the melanoma, may be ascribable to a greater proportion of cells actively undergoing DNA synthesis at any one time in the melanoma than in the EAT. Twelve hours after the injection of ara-C, the thymidine index of the B16 melanoma recovered

to the level of that of the control animals. By the 16 hour point, the thymidine index had surpassed the control values. This may be explained by a sudden release of cells that were blocked in the S-phase by ara-C (Karon and Shirakawa, 1969), and which now commenced DNA synthesis, thereby acquiring the thymidine label. This "over-shoot" may be explained simply since all cells not in the S-phase during the effective levels of ara-C continue to move around the cell cycle. In other words, the cells remained incapable under the action of the drug of completing the S-phase, and only after its effect diminished could they continue with the synthesis of DNA. As a consequence, a rapid increase in the thymidine index was observed in the B16 melanoma experiment, where the thymidine index suddenly increased from 0.82 to the high of 16.55 within twelve hours. A similar phenomenon, after the release from the effects of ara-C, was reported by Kim and Eidinoff (1965) in HeLa S-3 cells. The sudden increase in the thymidine index might represent a synchronization of the cell population. Following the "over-shoot" of the thymidine index, the latter soon declined to a level below the mean control value. A similar synchronization and "over-shoot" was observed in the EAT cells as well, but to a much lesser degree. The recovery of the thymidine index in the EAT cells was more gradual, and the experimental period under study was not sufficiently long to show a return of the index to the control levels.

The normal cell cycle time for the B16 melanoma was found to be approximately 14 hours, and about 54 percent of the time was taken up

by the S-phase. The estimate of 7.5 hours for the duration of the S-phase agreed with the findings of Helpap and Maurer (1967) who described the in vitro and in vivo (mice) duration of B16 melanoma to be seven hours. No other reference to the B16 melanoma cell cycle in mice could be found. According to the present work, the duration of mitosis equalled 1 hour and 20 minutes, the G_1-phase equalled 1 hour and 20 minutes, and the G_2-phase was 3 hours and 50 minutes.

After the treatment with ara-C, the cell cycle of the B16 melanoma changed considerably. The ascending limb of the first wave was begun but a plateau was reached only one hour after the injection of ara-C. This soon declined to a low of 14.68 percent of labeled mitoses seven hours after the injection of ara-C. Between seven and nine hours after the injection of ara-C, the ascending limb of the delayed wave began. It is interesting to note that the delay of 8.5-9.0 hours approximated the total of the S and G_2-phases (7.5 hrs. + 1.33 hrs.= 8.83 hrs.). The duration of this delay was understandable since ara-C prevented the entry of cells into the S-phase (Karon and Shirakawa, 1969). Those cells which had already started DNA synthesis and those in the G_2-phase were not affected by ara-C, and would have continued on into the mitotic phase. The number of labeled cells undergoing division during this time interval became smaller and smaller, as reflected by the decline of labeled mitoses. Since ara-C only had the ability to act for approximately one hour, subsequent to this, cells which were blocked at the beginning of S would have now proceeded to synthesize DNA. Those cells moving through the S and G_2 phases were consequently synchronized and entered mitosis simultaneously (Figure 7).

The duration of the S-phase was decreased from a 7.5 hour minimum in the untreated cell cycle to 6 hours in the treated tumor cells. This shortening of the S-phase in the treated cells may have been an attempt to shorten the overall cell cycle time so that new cells could be produced quicker to replace those killed by ara-C. The duration of the experiment was insufficient to obtain the second wave of labeled mitoses and consequently the duration of the cell cycle of the treated B16 melanoma could not be determined.

The cell cycle time of untreated EAT cells was similar to those reported by other authors, ie., 16 hours. The duration of the S-phase was determined to be 10 hours, while the mitotic duration was 2 hours and 15 minutes; G_2 phase equalled 2 hours and 30 minutes and the G_1 phase was 1 hour and 15 minutes. The durations of the cell cycle phases for the hypotetraploid strain of Ehrlich ascites tumor cells of Baserga (1963) and Baserga and Gold (1963) as listed in Figure 3 agreed well with these results. The reason for the agreement between the present study and previous studies with regard to the cell cycle and the disagreement between mitotic rates found by previous authors and this work can be explained due to technique. In ascertaining the cell cycle, only those cells in the various stages of mitosis both labeled and unlabeled were scored. In the colchicine series for the determination of the mitotic rates, both colchicine metaphases and interphase cells were counted. If a greater number of these interphase cells were unable to divide or even dying, then the counts would be erroneously low. Thus the cell cycle presented

here represents the normal cell cycle of those cells in the proliferating fraction of the Ehrlich ascites tumor population. However the proliferating fraction of this tumor population may differ from that in tumors previously used, and as a result the mitotic rates will be correspondingly different.

In the cell cycle of the treated EAT cells there was a similar delay as witnessed in that of the treated B16 melanoma. The ascending limb of the first wave only reached 20 percent of labeled mitoses before declining. The delay pattern in the treated EAT cell cycle was similar to that found for the B16 melanoma and its deviation (12 hours) again agreed with the duration of the S-phase (10 hours) and the G_2-phase (2 hours and 30 minutes). After this delay the ascending limb of the first wave was formed. The deviation of the S-phase in the cells treated with ara-C was 10 hours and thus was not altered from the S-phase duration of the normal cell cycle. Why there was no shortening of the S-phase in the treated EAT cells similar to that seen in the B16 melanoma can not be explained. It may be due to the different effects of ara-C upon different cell populations.

The purpose of the remaining experiments was to ascertain the effects of multiple doses of ara-C, within a 24 hour period, upon the mitotic rate of B16 melanoma and Ehrlich ascites tumor cells. A secondary consideration was the effect on the tumor cells, of allowing host recovery periods between the series of injections. The first three experiments dealt with the B16 melanoma while the last one involved the Ehrlich ascites

tumor.

The first experiment where four 50 mg/kg injections of ara-C were administered on the 6th and 10th days the mitotic rate of the B16 melanoma was reduced to 0.47 and 0.42 percent respectively. After the first three day host recovery period the mitotic rate returned to 7.11 percent. This level was similar to the mean mitotic rate found for the control group in experiment M1. This recovery indicated that the host recovery period of three days was too long, as it also allowed recovery of the tumor. Following the second injection series and rest period, the recovery was much less, with the mitotic rate increasing from 0.42 percent to 1.50 percent. Although the tumors appeared to recover slightly, only two host animals survived the second injection series and all the animals of the fifth group died during the second rest period. It can be concluded that this dosage of ara-C was too toxic for short interval injections within a 24 hour period and that a three day host recovery period was too long as it allowed the tumor to completely recover.

In the second experiment the dosage levels of ara-C were reduced to 25 mg/kg, and this resulted in mitotic rates of 0.97, 0.71 and 0.57 percent after injection series on days six, eight and ten, respectively. These values were not as low as those seen in the previous experiment where 50 mg/kg dosages of ara-C were used. However, the mitotic rates became progressively lower after each successive sequence of injections. The host recovery time in this second experiment was reduced to one day, and this allowed the mitotic rate to return to 1.40 and 2.78 percent, after the first and second recovery periods, respectively. However, once

again no animals survived the final rest period.

In view of this loss of animals receiving 25 mg/kg of ara-C, the dosage was again reduced to one half, ie., 12.5 mg/kg of ara-C. The mitotic rates were not now reduced to the levels seen in the two previous experiments. However, after the one day host recovery periods the mitotic rates of the tumor exceeded the control values previously obtained from experiment M1. This "over-shoot" possibly reflected the property of ara-C in low dosages to partially synchronize the tumor cell population. This level of ara-C administration showed no toxicity.

As the 25 mg/kg dosage level was the most effective in reducing the mitotic rate of the B16 melanoma, this dosage was also used against the Ehrlich ascites tumor cells. The mitotic rates were reduced after each 24 hour series of ara-C injections and there was little recovery during the host rest periods. However, one animal died in the sixth group and once again no animals survived the final rest period. This revealed that a 25 mg/kg dose of ara-C was still too toxic for the Connaught mice bearing the EAT cells.

It is obvious that more work must be undertaken to study the effects of various regimen of ara-C injections upon tumor populations. Skipper et al. (1967) demonstrated that 15 mg/kg doses administered at 3 hour intervals for 24 hours reduced a L1210 leukemia cell population substantially even with a 3 day rest period between each series of injections. However, they did not report how much of the population recovered and did not observe the mitotic rates. The host recovery time

between such multiple treatments seems to be extremely important as indicated in the present studies. Such a rest period allows the animal's bone marrow and gastro-intestinal mucosa to recover from the effects of ara-C. Kline et al. (1966) demonstrated that in several multiple schedules of ara-C treatment the effects on retarding leukemia growth as revealed by survival times, were similar as long as the time interval between treatments was less than seven days. Tyrer et al. (1967) observed that ara-C was more effective in increasing the survival time of mice bearing L1210 leukemia when 1, 3-Bis(2-chloroethyl)-1-nitrourea was used to pre-treat the cells. Such studies, however, do not really relate what is happening at the cellular level, such as the effects of ara-C on the mitotic rate or thymidine index.

One interesting study that should be investigated in this method of trying to provide a more efficient reduction of tumor cell populations, is the use of colchicine. It could be injected into the tumor bearing animal at a dosage which would synchronize the cell population, and then ara-C could be administered at precisely the right time to effectively kill all synchronized cells entering the DNA synthesizing phase. This has previously been investigated with some success, in an <u>in vitro</u> study on L1210 leukemia cells (Vallamudi et al., 1968).

Another approach which could be employed, and has been made more apparent by these studies, is the synchronous property of ara-C itself. It is possible that if a low dose of ara-C was first used to synchronize the cell population then larger doses could be used to kill those cells

entering DNA synthesis. This procedure would effectively eliminate a greater proportion of tumor cells, than would be killed in a non-synchronous tumor population.

CHAPTER VI.

CONCLUSIONS

1. A 50 mg/kg dose of ara-C both reduced the daily mitotic rate of the B16 melanoma tumor more effectively and its effects were more prolonged than a 80 mg/kg dose.
2. Ara-C had no apparent effect upon the transplantability of the B16 melanoma or the EAT cells.
3. Ara-C was shown to take effect within 15 minutes of its administration. Its maximum effect was seen 45 and 60 minutes after injection in B16 melanoma and EAT cells respectively.
4. Ara-C acts to sychronize a portion of the cell population in both the B16 melanoma and the EAT cells.
5. The durations of the phases of the cell cycle of the B16 melanoma tumor cells, on day 10 of tumor growth, were shown to be:- t_C = 14 hrs., t_s = 7 hrs. 30 min., t_{G2} = 3 hrs., 50 min., t_M = 1 hr. 20 min. and t_{G1}= 1 hr. 20 min.
6. The duration of the phases of the cell cycle of the Ehrlich ascites tumor cells were shown to be:- t_C = 16 hrs., t_s= 10 hrs., t_{G2} = 2 hrs. 30 min., t_M = 2 hrs. 15 min. and t_{G1} = 1 hr. 15 min.
7. The cell cycles of the B16 melanoma and the Ehrlich ascites tumor after ara-C treatment were both delayed by an interval of time euqal to the sum of the durations of t_s and t_{G2}. This would indicate that ara-C blocks the entry of cells into the DNA synthesis stage of the cell cycle.
8. There is some indication that ara-C treatment may decrease the duration of the S-phase in the B16 melanoma tumor cells.

9. Multiple injections of a 50 mg/kg dose of ara-C proved to be too toxic for the host animals, while an intervening rest period of 3 days allowed tumor as well as host recovery. Reducing the dose to 25 mg/kg and the rest periods to 1 day was more effective but still toxic. Even smaller doses (12.5 mg/kg) resulted in synchronization of the tumor cell population and this was not effective in combating the growth of the tumor.

ADDENDUM

The purpose of this addendum is to clarify and specifically relate further evidence on the action of two drugs, nitrogen mustard and actinomycin D.

Nitrogen mustard is not only a premitotic inhibitor of cells as mentioned by Layde and Baserga (1964) in work on EAT cells, but other effects on the cell generation cycle have been reported. Walker and Helleiner (1963) in an *in vitro* study of L-stain mouse fibroblasts demonstrated that nitrogen mustard was most effective on cells in the S phase, but less so on cells in the G_1 and G_2 phases. Bruce et al. (1966) have demonstrated that normal hematopoietic and transplanted lymphoma colony-forming cells, when treated with nitrogen mustard, were killed irrespective of the phase of the generation cycle.

The action of actinomycin D upon EAT cells, as described by Baserga et al. (1965), points to an inhibitory action upon G_1 cells. However, Bruce et al. (1966) concluded from their work on normal hematopoietic and transplanted lymphoma colony-forming cells, that actinomycin D killed cells in all or most of the cell generation cycle phases. Sensitivity of a cell population was shown to be dependent on the fraction of the total population in the proliferation state. More specifically, actinomycin D has been shown to inhibit the DNA-directed synthesis of RNA (Tsunamura and Sartorelli, 1966). The same authors also proposed that any pre-existing RNA was catabolized thus minimizing any conservation and utilization of these molecules for the survival of the cells.

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CHAPTER VII.

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