THE UNIVERSITY OF MANITOBA

CELL KINETICS OF B16 MELANOMA AND THE INDUCTION OF IN VIVO CELL SYNCHRONY BY CYTOSINE ARABINOSIDE

by

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To Trish and Ian.

ABSTRACT

B16 melanoma, a solid transplantable tumor, was employed as a model to determine the feasibility of producing in vivo cell synchrony by the use of the specific DNA-synthesis inhibitor - cytosine arabinoside (ara-C). The asynchronous tumor cell population grew rapidly during its early phase of growth but declined slowly in its later phases. The decline in the tumor growth rate was brought about by a marked prolongation of the total cell cycle time with age, apparently accompanied by an increasing cell loss largely due to a continuous expanding central necrotic area. The labeling, mitotic and degenerating indices of B16 melanoma did not reveal any diurnal variations. The mean growth fraction (GF) of the tumor, during the days 7 to 16 of tumor growth, was 0.53.

A single 50 mg/kg dosage of ara-C did not alter the total cell cycle time of B16 melanoma of day 6 and 18 tumors from that of the untreated tumor cell population. However, the duration of the S phase appeared to be shortened in the treated tumor cell population.

Different levels of cell synchrony were produced in vivo in the melanoma cell population, employing seven different ara-C regimens that varied in dose and injection schedules. The highest degree of cell synchrony was achieved by eight 12.5 mg/kg injections of ara-C administered at 2 hour intervals. In the latter series, the synchrony index (SI) was 40.7. The experimentally observed SI was then adjusted to the size of the GF of the tumor to 76.8, representing indeed a high degree of cell synchrony. It became evident from the experimental series that in order to achieve satisfactory cell synchrony in the tumor, the ara-C block must remain effective longer than 8 hours to allow all

cells present at the time of drug administrations in the latter part of S, in G_2 and M to accumulate at G_1-S juncture. There seemed to exist also a direct correlation between the degree of SI achieved and the proportion of cells killed by a prolonged block of DNA synthesis, the latter expressed by the degenerating index. Most of the cells killed by a prolonged DNA synthesis block were probably in the later phase of G_1 at the time of drug administration and died because of having been prevented for a prolonged period from continuing in the cell cycle.

The degree of cell synchrony attained with a particular ara-C schedule was subsequently reproduced in other experimental series, signifying that it is readily reproducible. It was ascertained further that in the synchronized tumor cell population, subsequently administered three 60 mg/kg injections of ara-C produced a greater degree of cell kill (DI) than a single 60 mg/kg injection.

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CHAPTER I

INTRODUCTION

PROBLEMS

The problems investigated in this study evolved as a natural extension of a previous study by the author (Gibson, 1969). The principal question that arose from that study was whether the low degree of cell synchrony produced by a single administration of cytosine arabinoside could be improved upon by regimen of multiple ara-C administrations. Therefore the principal object of this study was an attempt to achieve a high degree of cell synchrony in a malignant *in vivo* cell population.

Before this main problem could be approached additional information was required concerning the growth characteristics of the tumor model, the malignant B16 melanoma. For example, the size of the growth fraction, the labeling, mitotic and degenerating indices of the tumor had to be determined. Further, it had to be ascertained whether these indices were affected by diurnal variations. Moreover, the mean cell cycle times on different days of tumor growth had to be known to facilitate a more expedient interpretation of *in vivo* cell synchrony and evaluation of its magnitude.

At the outset of this investigation, in the fall of 1969, only one other report of an *in vivo* cell synchrony study existed in the literature, by Mauro and Madoc-Jones (1969). These authors had produced a minor degree of synchronization of lymphoma cells by a single administration of hydroxyurea, a procedure comparable to that in the previous studies in our laboratory employing cytosine arabinoside. Hence, chances appeared in favor that an enhanced *in vivo* cell synchrony could be produced by manipulating the ara-C dosage and the injection schedule of the drug. Therefore, the degree of cell synchrony achieved in the different series was employed as a means of comparison between

the various regimens in the course of production of cell synchrony. When this study was begun, reports on kinetic parameters of such partly synchronized cell populations did not exist. This lead to a further natural extension, the ascertainment of alterations in the cell kinetics of the B16 melanoma once it was synchronized, in comparison to its asynchronous growth characteristics.

In the last section of the present report the effects of additional administrations of ara-C on the previously in vivo synchronized B16 melanoma are discussed.

SIGNIFICANCE OF THE INVESTIGATION

The principal significance of the present study was to determine whether a fairly high degree of synchrony can be achieved in the cell population of a solid in vivo tumor, and if so, to elucidate its kinetic parameters. In vivo cell synchrony studies of this type remain still in their infant stages. The only comparable investigation available at present is that by Rajewsky (1970) who succeeded to produce in vivo cell synchrony in a rat sarcoma by employing hydroxyurea. That author stated: "Although cell populations synchronized in culture are preferable for technical reasons, they evidently lack many characteristics of the highly controled cell systems in the intact organisms. It would, therefore, be of interest if cell systems were available with a satisfactory degree of synchrony under in vivo conditions.".

Synchronization of any in vivo tumor cell population may render it more susceptible to the available chemotherapeutic methods. It may allow in addition other anticancer drugs to be tested to enhance their effectiveness. Any further possible benefits that in vivo cell synchrony may offer will have to be ascertained by the clinical chemotherapist.

CHAPTER II

REVIEW OF THE LITERATURE

TUMOR GROWTH

During the past decade, the study of tumor growth has been extensive and a great deal of new information was ascertained. As this work deals with a solid tumor, B16 melanoma, most of the information presented in the following, reviews the growth of solid tumors. However, reference is appropriately made to ascitic and leukemic tumors.

It was demonstrated that most tumors, perhaps even all tumors, are composed of mixed fractions of cells. The tumor fractions are classified by the circumstance that some of the cells divide, thus comprising the proliferating fraction, while other cells never or rarely divide and constitute a nonproliferating fraction of the tumor cell population. The nonproliferating cell fraction, is not only comprised of nondividing cells, but also tumor cells which are either dying or in the so-called G_0 phase. The possible fractions of a tumor population are presented in Chart 1. Each of these cell fractions was demonstrated to constitute an inherent part of the growth characteristics of a particular tumor, and each may have a different influence on the growth characteristics of a particular tumor, depending on the proportion of cells participating in each of these fractions.

It is the predominant feature of all tumors that the cells of the proliferating fraction divide and give rise to new tumor cells. The growth of a tumor population has been shown to be dependent on the fact that more tumor cells are produced in the cell population than became lost from it.

Chart 1.

Possible fractions of a tumor population. The squares represent the nonproliferating fractions of a tumor population. The circle represents the circulating proliferating fraction. The arrows indicate possible fates of each fraction. G_1 = post-mitotic phase; S = DNA synthesis phase; and G_2 = pre-mitotic phase.

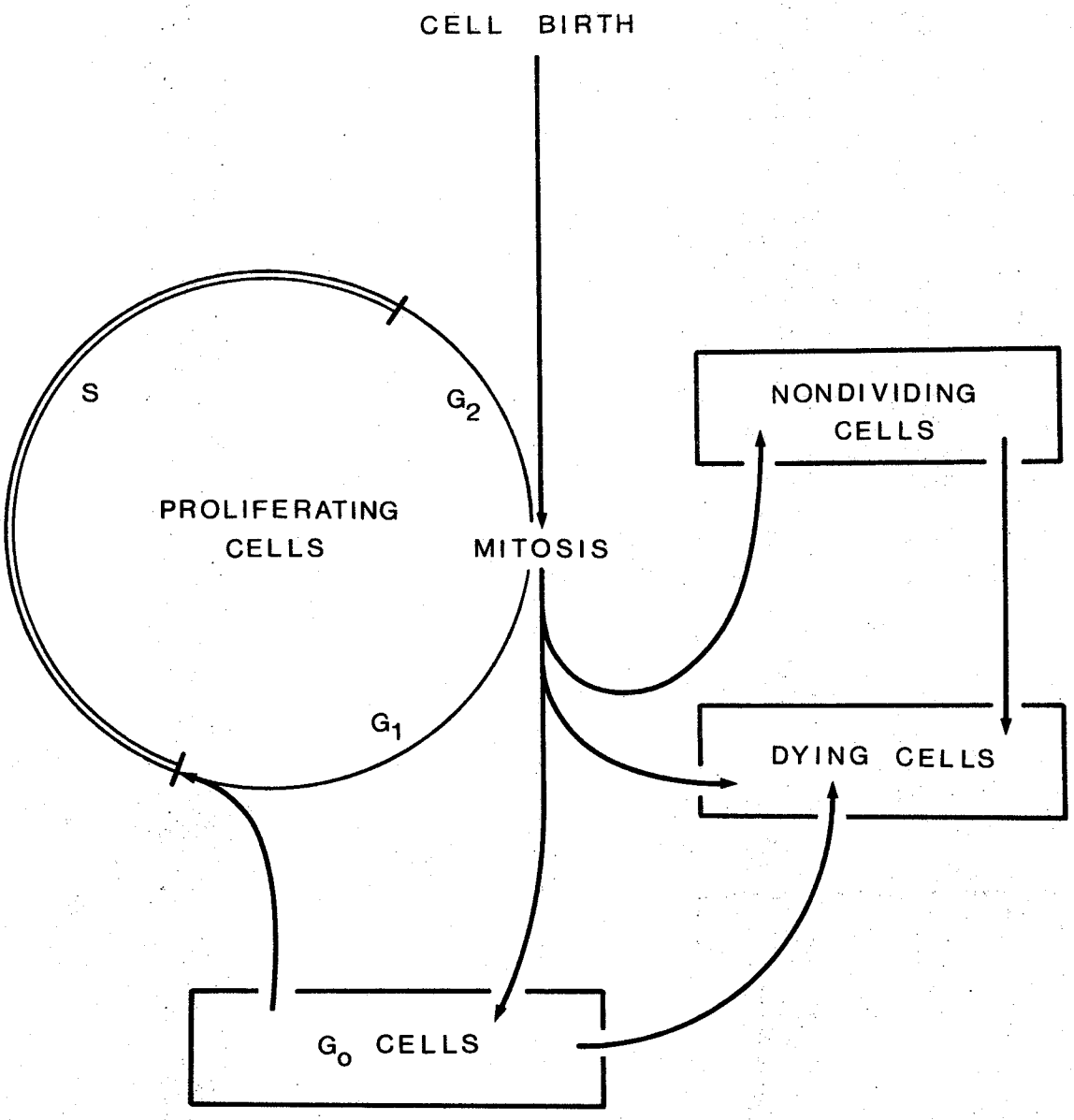

CELL BIRTH
S
G2
PROLIFERATING CELLS
MITOSIS
G1
NONDIVIDING CELLS
DYING CELLS
Go CELLS

Chart 1

Growth curves for various tumors have been developed by measuring the accumulation of tumor cells by estimating the cell number, tumor weight or tumor volume as related to time. Most tumors, both ascitic and solid tumor forms, grow rapidly during the early stages of their development, and as time progresses, a gradual deceleration of growth occurs (Laird, 1964, 1965; McCredie et al, 1965). The growth of Krebs and Ehrlich ascites tumors best fit an exponential equation (Patt and Blackford, 1954; Baserga, 1964); however, the growth rate of these tumors does not follow this equation throughout their entire growth period. In fact, only brief segments of the growth curve will fit such an equation. The growth curve for most solid tumors can be algebraically expressed by a Gompertzian function (Laird, 1964, 1965). This function has been described by a curve which was exponential during the early growth stages but tends to level off or decelerate during the latter stages of tumor growth. The leveling off of the growth curve was attributed to a number of retarding factors affecting tumor growth. Such factors may be an increase of the average generation (cell cycle) time, a decline of the growth fraction, or a loss of proliferating cells as time progresses (Dethlefsen et al, 1968; and Laird, 1969). Tannock (1970) suggested that the different rates of proliferation between parenchymal and stromal cells in mouse mammary gland tumors may also be a major cause for the slowing of tumor growth. Another factor which may produce similar effects may be the host's defence mechanism against the growth of such tumor cells (Dethlefsen et al, 1968).

Many authorities in the field observed that the generation time of proliferating tumor cells was relatively independent of tumor size

(Mendelsohn, 1965), and also that it did become altered as the overall rate of tumor growth was undergoing the Compertzian retardation (Frindel et al, 1967). An increase in cell cycle time with the age of the tumor was demonstrated in Ehrlich ascites (Steel and Lamerton, 1969; and Harris et al, 1970) adenocarcinoma and plasmacytoma (Simpson-Heren and Lloyd, 1970) tumor cells. Consequently, in some tumor populations the increase in the generation cycle of tumor cells definitely contributes to the retardation of tumor growth.

The growth fraction, as defined by Mendelsohn (1962), indicates the proportionality of tumor cells within the proliferating compartment of a tumor. The growth fraction of some tumors declined with the age of the tumor. For example, Lala (1968) observed in Ehrlich ascites tumor that the growth fraction declined from 0.82 on the first day of tumor growth to 0.53 on the seventh day, while Frindel et al (1967), reported that in a transplantable mouse fibrosarcoma the growth fraction decreased from 0.40 to 0.24 on days 3 and 20 of tumor growth, respectively. In contrast, it was also observed that the growth fraction of C3H mouse mammary tumors remained relatively stable with time (Mendelsohn, 1962).

Perhaps the most significant parameter, in tumor kinetics, responsible for the retardation of tumor growth is the amount of cell loss from the proliferating fraction of a tumor cell population. Evidence has been provided by studies on Ehrlich ascites (Edwards et al, 1960; Baserga, 1963; and Kim and Evans, 1964), transplantable fibrosarcoma (Frindel et al, 1967) and a mammary gland adenocarcinoma (Clifton and Yatvin, 1970) tumor cells, that cell loss plays a less significant role in recently transplanted tumors as compared with

tumors that had been growing for some time. In a study of the C3H mouse mammary tumor, the rates of cell death were determined to be as high as 80% (Mendelsohn and Dethlefsen, 1968). A 'cell loss factor' affecting many tumors was demonstrated, and this factor was responsible for the discrepancy between the potential doubling time and the actual volume doubling time of the tumor cell population (Denekamp, 1970; and Looney et al, 1971). The 'cell loss factor' of many rodent tumors, as listed in a report by Denekamp (1970), ranged from 0.00 in a rat sarcoma to 0.93 in a hamster carcinoma. Similarly, Owen and Steel (1969) observed in spontaneous tumors of domestic animals (dogs and cats) that extensive cell loss occurred. Steel and Lamerton (1966, 1969) summarizing the work of other authors, concluded that in the majority of human tumors, cell loss could be the dominant factor determining the growth rates.

The mechanisms whereby cells become lost from tumors are: metastasis, exfoliation, and cell death in situ. Metastasis is the process where tumor cells migrate to other parts of the body. The pathways may be via the blood stream, lymphatic channels, or localized tissue spreading. However, so far direct evidence was not provided that the loss of tumor cells by metastasis was a dominant factor affecting the growth rate of tumors, apart perhaps, in tumors deriving from the lymphoid system. Cell loss, brought about by exfoliation, is a significant factor in superficial carcinomas of the gastrointestinal tract, for instance, but there is little direct evidence that this process occurs in other types of tumors as well. Cell death in situ appears to be the most decisive factor affecting tumor growth. In tumors which tend to become grossly necrotic, continual cell death

occurs with a concurrent enlargement of the necrotic mass. It was suggested that cell death and the production of necrotic areas, was ascribable to a state of poor nutrition of the tumor over prolonged periods (McCredie et al, 1965; and Steel, 1968). Yet, Laird (1969) denies that the failure of the blood supply to provide oxygen and nutrients to the tumor tissue was the principle cause for the growth retardation of tumors. Instead, that author asserted that growth retardation was a metazoan characteristic of organisms and their constituent parts, which included tumors and that tissues retained this basic genetically programmed process even when they became malignant.

Another process of cell death seemed possible by the presence of isolated degenerated nuclei, but remote from the regions of necrosis. Mendelsohn (1960) encountered such degenerated nuclei in the C3H mammary tumors; their numbers ranged from 0.8 - 1.7%. Steel (1968) believed that death of these cells probably represented a 'mitotic death'.

In summary, the growth characteristics of a particular tumor has to be examined very closely, because a number of factors can possibly affect the growth of that tumor population. In general, the cell cycle time, growth fraction, and the amount of cell loss from the proliferating population lend the tumor its specific growth characteristics. Such a dominant factor of a tumor cell population must be ascertained prior to performing any experiments with the particular tumor. It should be kept in mind that serially transplanted tumors, after a limited number of passages can, with time, change their cell cycle duration, growth fraction and the proportion of cell loss. For instance, Begg (1971) observed recently that in NT1 carcinoma in C3H mice, both

the cell cycle and volume doubling times became prolonged between the 17th and 27th passages.

Chart 1 presents another yet theoretical cell fraction, called the 'G_o cell' (Quastler, 1963). This fraction is believed to be composed of cells that retained their potentiality of division although they rarely divide. It is assumed that this cell fraction can act as a 'safety measure' to repopulate a tumor population if its proliferating population is destroyed. However, little information beyond the theoretical stage is yet available. Steel and Lamerton (1969) differentiate between the G_o cell of normal tissues, such as the liver, and the G_o cell thought to exist in tumors. They assumed that the stimulus of a G_o cell to undergo proliferation in the liver or any other normal tissue was controlled by factors governing normal cell proliferation, whereas the release of the G_o cell was not under a similar control. For instance, environmental factors, such as hypoxia, may prevent G_o cells from entering the proliferative cell cycle for long periods of time. However, in both cases, normal or abnormal, the G_o cell retains its proliferative potential.

Inasmuch as tumor growth is highly dependent on the number of cells being produced, some of the characteristics of the proliferating cell fraction have to be examined more closely (Chart 1).

One measure employed to indicate the degree of mitotic activity of a tissue is the mitotic index. It represents the number of cells in the mitotic phase (undergoing division) at any instant of time among 1000 cells. This index is most useful for comparing the mitotic activity of several tissues or of one and the same cell population during different experimental conditions.

An extension of the mitotic index is the mitotic rate, ascertained by the cytostatic drug colchicine. Although colchicine halts the normal progression of mitotic cells in the metaphase stage, it does not alter the flow of cells into mitosis (Stevens-Hooper, 1961). Using the proper dosage (Bertalanffy and Leblond, 1953; and Wallace, 1964) of colchicine, the proportion of colchicine metaphases arrested during either a four or six hour period yields the mitotic rate of a tissue. The mitotic rate is the percentage of cells undergoing mitosis during a certain time interval. The daily mitotic rate is determined by ascertaining the percentage of cells entering mitosis during a 24 hour period. From the latter data, the turnover time, the interval required for the division or replacement of 100% of the cells, can be calculated (Leblond and Walker, 1956; and Stevens-Hooper, 1961).

The daily mitotic rates of various mouse and rat tumors ranged from 34.0 - 62.8% on different days of tumor growth (Bertalanffy and Lau, 1962; Bertalanffy and McAskill, 1964a,b; and Bertalanffy et al, 1965). None of the above mitotic rates exhibited any diurnal variations. In the course of these studies, the rates of cell production in neoplasms were compared with those of normal renewing cell populations; it became evident that some tumors proliferated more slowly than many normal tissues (Bertalanffy, 1967).

From the mitotic index and the mitotic rate, the mitotic duration can be calculated (Leblond and Stevens, 1948; and Quastler and Sherman, 1959). The mitotic duration of many tumors ranged between 30-90 minutes, with a predominant duration of roughly 60 minutes (Denekamp, 1970; and Simpson-Herren and Lloyd, 1970).

A further indicator of tumor proliferation velocity is the labeling index. It represents the percentage of cells that incorporate H^3-thymidine during a given interval of time, usually ranging between 15-60 minutes. This index represents that proportion of cells undergoing DNA synthesis during the time H^3-thymidine is available to the tumor population (Messier and Leblond, 1960; and Baserga and Kisieleski, 1962). The tracer, H^3-thymidine, was not incorporated into cells undergoing DNA synthesis for much longer than 1 hour after its administration (Blenkinsopp, 1968). The labeling index ranged in various rodent tumors from 16 to 69% (Tannock, 1969; and Denekamp, 1970). Moreover, the proportion of cells passing through the S phase of the proliferative cycle remained constant over brief periods of time (24-48 hours) in asynchronous tumor cell populations (Bertalanffy and Gibson, 1971). Notwithstanding, the labeling index decreased as some tumors became older (Tannock, 1969; and Simpson-Herren and Lloyd, 1970).

An important aspect of any tumor analysis is the determination of the phases of the cell cycle (Chart 1). The cell cycle was initially defined by Howard and Pelc (1951) to be the orderly sequence of metabolic activities, from the midpoint of mitosis to the midpoint of the successive mitosis. There are two segments of the cell cycle, one is the visible appearance of one of the four classic phases of mitosis, as described by Leblond and Stevens (1948), and the second is the interphase between two successive mitoses. Interphase has itself been divided into three segments, chiefly by the identification of the S phase employing H^3-thymidine (Chart 1).

Between mitosis and the S phase lies the first (postmitotic)

gap or G_1, while the second (premitotic) gap, G_2, intervenes between the S phase and mitosis. Protein and RNA synthesis occur throughout most of the cell cycle, but fluctuates quantitatively as the cells pass through the various phases. In contrast DNA synthesis becomes synthesized during the S phase exclusively. The chemistry of the cell cycle was reviewed in detail by the articles of Prescott (1969) and Baserga (1968, 1969).

The mean durations of the individual phases of the cell cycle of any asynchronously growing populations can be determined by combining two positive observations, DNA synthesis and mitosis, to a labeled mitoses curve (Quastler and Sherman, 1959). At zero time, H^3-thymidine, a specific DNA precursor label, is administered. The label is incorporated by those cells engaged in DNA synthesis. If tumor bearing mice, for instance, receive the H^3-thymidine tracer, and are killed in groups at intervals during a period equaling twice the estimated cell cycle duration, the labeling index of the mitotic figures, as observed by autoradiography, will rise as soon as the cells, that were in the S phase while the label was available, passed through G_2 and entered mitosis. This permits the measurement of the length of the G_2 phase. The curve of the labeled mitoses should ideally rise to 100%, if all cells of the population are within the proliferating fraction. The curve should remain at that plateau for a period equalling the length of the S phase, and then decline when all labeled cells have passed through mitosis. Cells which were in the G_1 phase while the pulse label was available will remain unlabeled and when those cells divide, the labeled mitoses curve will equal zero. Barring toxic effects of H^3-thymidine, labeled cells, occuring as labeled mitoses in the first

wave will redivide after some time, producing a second wave. The phases will again be delimited in the order G_2, S and G_1. The time elapsing between the 50% of mitoses being labeled in the first and second ascending limbs of the labeled mitoses curve signifies the duration of the mean cell cycle time.

For a more detailed discussion and review on these problems the excellent work by Lala (1971) should be consulted.

B16 MELANOMA

This tumor, more correctly identified as a carcinomatous B16 melanoma, originally arose spontaneously on the ear of a female C57BL/6J mouse in 1954. Since then, it has undergone innumerable transplantations.

The tumor is a black (melanin) mass, encapsulated by a thin connective tissue layer (Green, 1968). The histology of the tumor was described both with the light (Bertalanffy and McAskill, 1964a; Gibson, 1969) and electron microscope (Demopoulos et al, 1965; Hu, 1971). The B16 melanoma consists of a very homogeneous parenchyma, composed chiefly of pigmented and nonpigmented tumor cells, and an occasional migratory leukocyte, fibroblasts and a network of capillaries.

Five days after subcutaneous transplantation, the tumor mass became palpable (Demopoulos et al, 1965). Two weeks after transplantation central necrosis was well advanced; the mice usually died 3-5 weeks following transplantation. Bertalanffy and McAskill (1964a) using the colchicine technique did not observe any significant difference between the mitotic rates of tumors on days 10 and 14 of tumor growth. They determined that 34 - 36% new cells were added

daily to the B16 melanoma, implying an extrapolated doubling time of 2.8 days. Likewise, with the colchicine technique, Gibson (1969) observed a daily mitotic rate of 46% on day 10 of tumor growth, and a doubling time of 2.2 days. In neither study were diurnal fluctuations of the mitotic activity observed of the B16 melanoma (Bertalanffy and McAskill, 1964a; Gibson, 1969). The former investigators ascertained further that metastases in the liver and spleen of that tumor exhibited similar mitotic rates as the primary tumors.

The duration of the S phase of the B16 melanoma, both *in vivo* and *in vitro* studies, was first reported by Helpap and Maurer (1967) to be 7 hours; yet, the stage of tumor growth was not indicated. More recently, by means of the percent labeled mitoses curve, the mean *cell cycle* time of B16 melanoma on day 10 of tumor growth was determined to be 14 hours (Gibson, 1969; Bertalanffy and Gibson, 1971). The durations of the cell cycle phases were: t_{G2}, 1.3 hours; t_S, 7.5 hours; t_{G1}, 3.8 hours and t_M, 1.4 hours.

CYTOSINE ARABINOSIDE

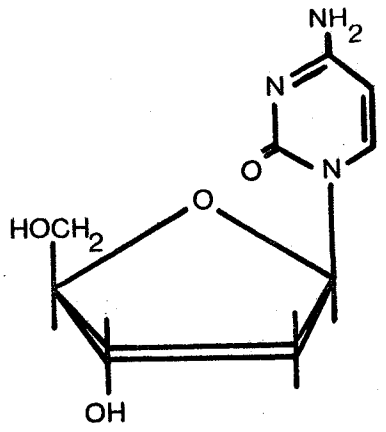
Cytosine arabinoside (1-β-arabinofuranosylcytosine, ara-C cytosar or cytarabine) is a synthetic, cycle specific nucleoside with both antitumor and antiviral activities. The nucleoside was independently synthesized by both Walwick et al, (1959) and Hunter (1965). Its chemical configuration is very similar to that of cytidine and deoxycytidine (Chart 2); however, the hydroxyl group on the C_2 of the sugar lies in a cis position to the glycosyl linkage rather than in the trans position. Two reviews in particular exist on biochemical and biological studies of the D-arabinosyl nucleosides, with special reference to ara-C (Cohen, 1966; Smith, 1966).

Ara-C has been observed to inhibit the proliferation of DNA viruses (Renis and Johnson, 1962; Underwood, 1962; Buthala, 1964; Prince et al, 1969; Renis, 1970) and bacteria (Pizer and Cohen, 1960; Slechta, 1961). A similar effect on mammalian cells is in its inhibiting action on DNA synthesis of normal and tumorous cell populations.

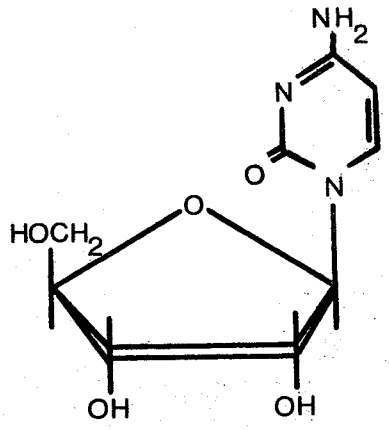
It became well established by many investigators that ara-C was a growth inhibitor of ascitic, leukemic and a variety of solid tumors in rodents and man. In vitro studies included cells from tumors such as leukemia of mice (Bach, 1969; Bodey et al, 1968; Chu and Fischer, 1965, 1968a,b) and man (Inagaki et al, 1969; Chan, 1969), murine tumors, such as Don C and KB cells (Karon and Shirakawa, 1969), 1970), L-cells (Graham and Whitmore, 1970a,b) and HeLa cells (Kim and Eidinoff, 1954). In vivo studies were performed on a variety of mouse leukemias (Chu and Fischer, 1962; Evans et al, 1964b; Hoffman et al, 1969; Kline et al, 1968; and Mizuno and Humphrey, 1969). Taper liver

Chart 2.

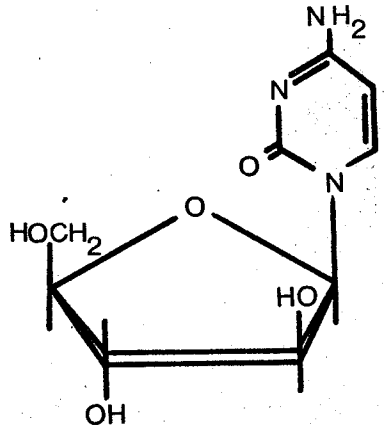
Chemical structures of cytosine nucleosides.



Deoxycytidine



Cytidine



1-β-D-arabinofuranosyl Cytosine

Chart 2

tumor (Cappuccino and Balis, 1969), mastocytoma (Chu and Fischer, 1962), murine Ehrlich ascites tumor (Kimball et al, 1966; Bertalanffy and Gibson, 1971), solid Ehrlich carcinoma (Evans et al, 1964b; Prince et al, 1969), murine sarcoma 180 (Mizuno and Humphrey, 1969; Evans et al, 1964), B16 melanoma (Bertalanffy and Gibson, 1971), rat Walker tumors (Wodinsky and Kenster, 1965), and a variety of human tumors transplanted into hamsters (Goldenberg et al, 1968a). Among these, an interesting observation reported by Cappuccino and Balis (1969) was that a statistically greater retardation of growth by ara-C existed in female mice bearing Taper liver tumors than in male mice bearing the same tumor, although the host toxicity was the same in either sex.

The inhibition of DNA synthesis in normal body tissues has by now been well documented. The tissues most severely affected by the drug are those undergoing continuous cell proliferation, for instance for renewal, because some proportions of their cells are always engaged in DNA synthesis, and thus are vulnerable to the inhibitory effect of ara-C. Reports on the inhibitory effect of DNA synthesis by ara-C exist for small intestine, lymph nodes, spleen, thymus (Leach et al, 1969; Lenaz et al, 1969; Gibson, 1969), and bone marrow (Leach et al, 1969; Papac et al, 1965) of mice. Also, the same tissues, including regenerating liver, were demonstrated to be affected by the inhibitory action of ara-C in the rat (Lenaz and Philips, 1970). Normal rabbit kidney cells in culture are likewise inhibited from doubling their DNA (Kaplan et al, 1968). The effect of ara-C on the crypts of the small intestine was the appearance of severe atrophy and degenerative changes (Lenaz et al, 1969; Leach et al, 1969). Karyorrhectic cells were found in lymph nodes of mice but **not** in the spleen or

thymus, the latter exhibiting less severe pathological alterations. The extent of such changes was believed to be related to the duration of DNA inhibition by the drug (Lenaz et al, 1969). Megaloblastosis occurred in the bone marrow where the granulocytes were first affected and only after a prolonged inhibition by ara-C, the other blood elements became influenced (Leach et al, 1969). Similar effects have been reported in bone marrow of patients with therapeutic dosages of ara-C (Bell et al, 1966).

Ara-C was demonstrated to be a potent immunosuppressive agent in the mouse, inhibiting the primary response to sheep erythrocytes (Fischer et al, 1966; Gray et al, 1968; Harris and Hersh, 1968) and bovine gamma globulin (Buskirk et al, 1965). The immunosuppressive activity of high dosages of ara-C were manifested in the rat (Mitchell et al, 1969a) by a decreased synthesis of γG and γM. Ara-C exerted similar effects on the suppression of antibody synthesis in man (Kaplan et al, 1966; Mitchell et al, 1969a). It is conceivable that the immunosuppressive activity of ara-C may lie in its ability to block macrophage production, a prerequisite for the subsequent uptake and processing of antigen.

The first indication that ara-C produced chromosome aberrations was provided in cultured human leukocytes where chromosome breakages resulted in an interchange of chromosomal segments (Kihlman et al, 1963). These investigators ascribed the chromosomal breakages to the inhibitory action of ara-C upon DNA synthesis. Similar observations were reported by Tally and Vaitkevicius (1963) in the bone marrow of patients treated with ara-C. However, Brewen (1965), Brewen and Christie (1967), and more recently Brewen and Fitzgerald (1968), studying human leukocytes, suggested

that the chromosomal aberrations were not brought about by the inhibition of DNA synthesis, but rather were independent. They based their conclusion on the observation that a high proportion of mitotic figures displayed abnormal chromosomes merely 3 hours after ara-C administration. They deduced that such breakages were produced in G_2 cells that had completed DNA synthesis prior to the treatment of ara-C. Block et al, (1965) and Bell et al, (1966) observed chromosomal changes even in nondividing cells of the marrow erythroid series; such were absent in the granulocytic precursors or megakaryocytes, however. These aberrations consisted of chromatid breaks both at the centromeric region or throughout their entire length, despiralization and extensive fragmentation. Nonetheless, following removal of ara-C from the media, both morphologic and cytogenetic abnormalities of the chromosomes rapidly returned to normal. That return to normality was attributed to the circumstance that ara-C was incorporated into the DNA molecule, and that ara-C became quickly deaminated to inactive forms. Similar effects have been demonstrated in WI-38 cells of human embryonic lung (Heneen and Nichols, 1967).

More recently, ara-C was demonstrated to produce mutant strains of L1210 cells, which were sufficiently different to render ara-C ineffective in prolonging the life of mice carrying the mutant leukemia (Bach, 1969). The mutation frequencies were in the range of 10^{-4} per cell per division cycle; the appearance of those extremely high frequencies produced by ara-C could not be explained.

It was observed further that ara-C produced chromatid breaks in the G_1, S and G_2 phases of the cell cycle (Benedict et al, 1970). Moreover, the inhibition of DNA synthesis by ultraviolet light reduced

the formation of such breakages (Benedict and Karon, 1971). This lent support to the concept that DNA synthesis in some form is not solely defined to the S phase and that semiconservative or "unscheduled" DNA synthesis was involved in the mechanism of chromatid breakage.

Ara-C induced cerebellar hypoplasia in newborn hamsters (Fischer and Jones, 1965) and acted as a teratogen in other species. Karnofsky and Lacon (1966) reported that ara-C produced more abnormalities in younger chicks (day 4) than in older ones (day 8). In the Wister rat, the period most critically affected by ara-C was between the 10-12 day of gestation when malformations were plentiful after drug administration to the mother animals, whereas none were produced in rats pregnant for 5-9 days (Chaube et al, 1968). In these rat studies, deoxycytosine (CdR), when it was given within 10 minutes of a single ara-C injection, provided complete protection against the teratogenicity of ara-C, H^3-ara-C was demonstrated to cross the placental barrier 1-2 hours after its injection, and about 2.5% of the radioactivity was detected in DNA (Chaube et al, 1968). In both instances, the teratogenicity of ara-C in the chicks and the rat fetuses, was attributed to the inhibitory effect of ara-C upon DNA synthesis.

Yet, this explanation may not be as clear cut, because Ritter et al, (1971) demonstrated in the rat that the actual cumulative depression of DNA synthesis, reflected both by the mean level and duration of depression after a given dosage, was more closely related to the embryotoxicity than to the post-treatment interval or the overall duration of depression caused by any one dosage. For example, these authors (Ritter et al, 1971) observed that a 25 mg/kg injection of ara-C did not exert any teratogenic effect, yet reduced DNA synthesis

by 78% of the control value, whereas a 200 mg/kg dose of ara-C was severely teratogenic but reduced DNA synthesis merely to 44% of the control value. Therefore a cause-and-effect relationship between the inhibition of DNA synthesis and teratogenesis cannot be supported by those data. Retardation of growth in fetuses, particularly with large doses, could be attributed to a balanced delay in the development schedule contingent on proportional slowing or transitory arrest of DNA synthesis in all tissues; specific malformations are less readily explained in these terms.

As mentioned earlier, the effects of ara-C upon viruses, bacteria, tumors and on normally proliferating cell populations were all related directly or indirectly to its inhibiting action on DNA synthesis. However, the precise mechanism of how ara-C inhibits DNA synthesis still remains to be elucidated. The metabolism and the principal sites of ara-C action are illustrated in Chart 3.

Ara-C, like the other pyrimidines, was taken up into cells either by inactive transport or facilitated diffusion (Jaques, 1962; Schrecher and Urshel, 1968). The net uptake of ara-C against the concentration gradient was due to removal of the compounds from the intracellular phase by conversion to metabolites. Kessel et al, (1967) claimed that the net uptake of ara-C was not impaired in drug resistant cell lines; however, Bach, (1969) indicated that there may in fact be a difference in the permeability of the cells to nucleosides.

Ara-C becomes detoxified to uracil arabinoside (ara-U) and ammonia in many cells and animal species: bacteria (Pizer and Cohen, 1960), mice (Camier and Smith, 1965), rat, dog, hamster and monkey (Mulligan and Mellett, 1968), mammalian cultures (Smith et al, 1965) and man

Chart 3.

Metabolism and sites of ara-C action.

Explanation of Abbreviations:

ara-C - cytosine arabinoside
ara-U - uracil arabinoside
DNA - deoxyribonucleic acid
RNA - ribonucleic acid
CDP - cytidine diphosphate
(P) - phosphorylation
dCDP - deoxycytidine diphosphate
dCTP - deoxycytidine triphosphate
ara-CDP - arabinosylcytidine diphosphate
ara-TCP - arabinosylcytidine triphosphate
CdR - deoxycytidine

References:

1. Bach, 1969
2. Camier and Smith, 1965
3. Cardeillac, 1964
4. Chu and Fischer, 1962
5. Chu and Fischer, 1965
6. Chu and Fischer, 1968a
7. Cleaver, 1969
8. Creasey et al, 1966
9. Dollinger et al, 1967
10. Furth and Cohen, 1968
11. Graham and Whitmore, 1970b
12. Jacques, 1962
13. Karon et al, 1966
14. Kessel, 1967
15. Kessel et al, 1967
16. Kim and Eidinoff, 1965
17. Kimball et al, 1966
18. Momparlor et al, 1968
19. Schrecker, 1970
20. Schrecker and Urshel, 1968
21. Silagi, 1965

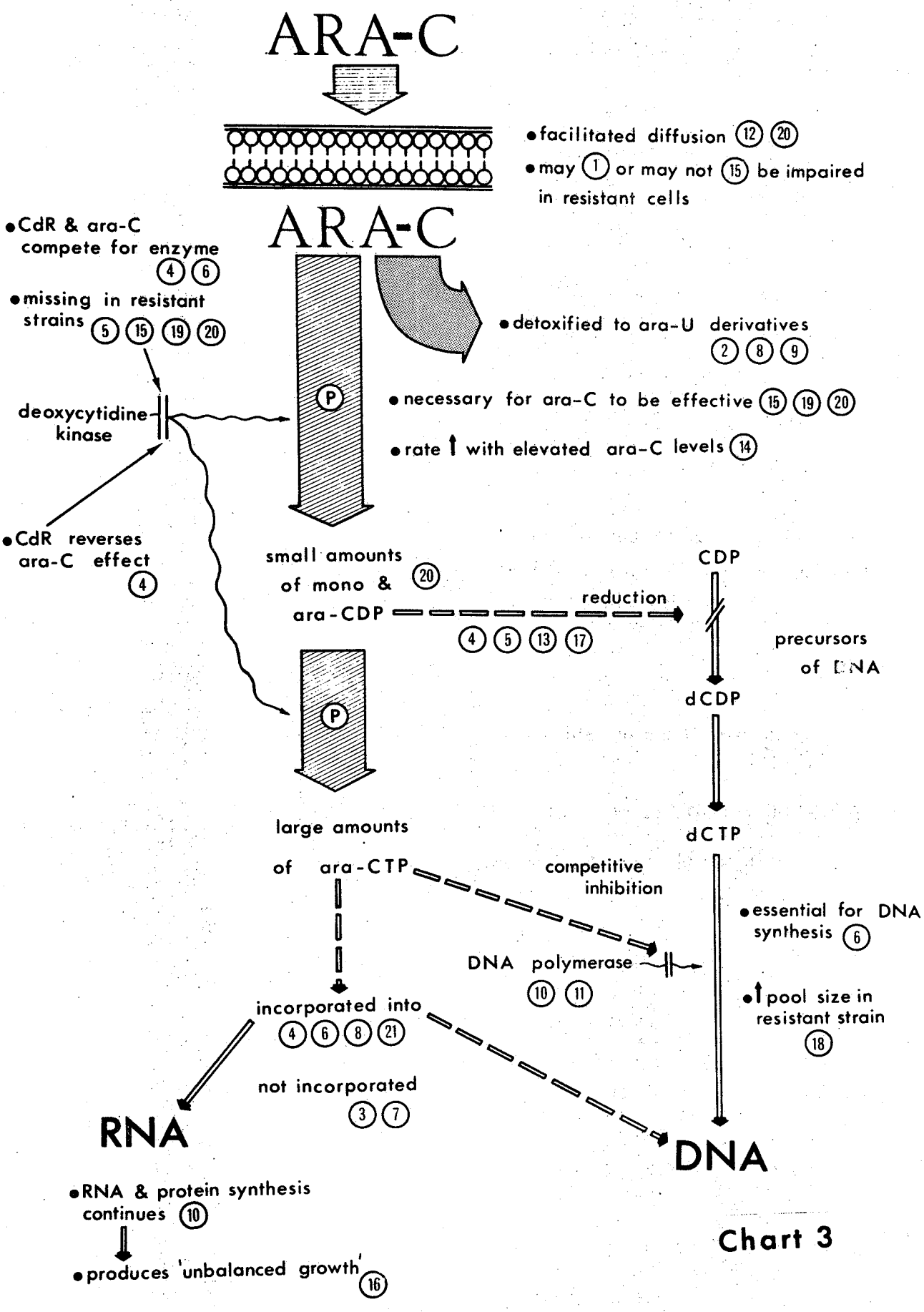

ARA-C
• facilitated diffusion (12) (20)
• may (1) or may not (15) be impaired in resistant cells
ARA-C
• CdR & ara-C compete for enzyme (4) (6)
• missing in resistant strains (5) (15) (19) (20)
• detoxified to ara-U derivatives (2) (8) (9)
deoxycytidine kinase
P
• necessary for ara-C to be effective (15) (19) (20)
• rate ↑ with elevated ara-C levels (14)
• CdR reverses ara-C effect (4)
small amounts of mono & ara-CDP (20)
reduction (4) (5) (13) (17)
CDP
precursors of DNA
dCDP
dCTP
large amounts of ara-CTP
competitive inhibition
• essential for DNA synthesis (6)
DNA polymerase (10) (11)
• ↑ pool size in resistant strain (18)
incorporated into (4) (6) (8) (21)
not incorporated (3) (7)
RNA
DNA
• RNA & protein synthesis continues (10)
• produces 'unbalanced growth' (16)

Chart 3

(Creasey et al, 1966). The specific site for the detoxification of ara-C was at a cellular level. It was deaminated by hydrolytic cleavage of the 4-amino group of the cytosine containing materials, yielding ammonia and corresponding uracil derivatives (Camier and Smith, 1965). The human liver and mouse kidney systems were actively engaged in this ara-C deamination in the entire animal (Dollinger et al, 1967), while in human leukemic cells intracellular deamination of ara-C occurred (Steuart et al, 1971). However, dCR deaminase, the deactivator of ara-C was present at a low level in normal mouse tissues and could not be traced in tumor cells (Ho, 1970). This observation was a direct contradiction of Steuart's work (Steuart et al, 1971). The serum half-lives of ara-C in various species was used as an indicator of the activity of the deaminase enzyme in a particular species. Mulligan and Mellett (1968) reported that after a 50 mg/kg (i.p. or i.v.) injection of ara-C the serum half-lives were 95, 43, 37, 35, 12 minutes in the dog, rat, mouse, hamster, and human respectively. No trace of ara-C was found in the serum of the monkey. Pretreatment of the dog and monkey with 100 mg/kg of tetrahydrouridine, an inhibitor of the deaminase, resulted in a doubling of the half-life of ara-C in the dog, and a half-life value for ara-C in the monkey approximating 150 minutes (Mulligan and Mellett, 1968).

Once it has gained entry to the cell, ara-C underwent phosphorylation to the various nucleotides of ara-C (Chart 3). Small amounts of mono- and diphosphate, and larger quantities of the triphosphate derivatives of ara-C were formed in L1210 cells by deoxytidine kinase (Schrecker and Urshel, 1969; Schrecker, 1970). The rate of the nucleotide formation increased with elevated ara-C

levels in both human and mouse leukemias (Kessel, 1967). It was proposed by several authors that the phosphorylation of the nucleoside ara-C was the key for its anti-DNA synthesis effects in tissues; and the lack of deoxycytidine kinase, the enzyme responsible for phosphorylation, would render the cells resistant to ara-C (Schrecker and Urshel, 1968; Schrecker, 1970; Kessel et al, 1967). Early studies dealing with ara-C indicated that deoxycytidine (CdR) reversed the inhibitory effect of ara-C on DNA synthesis (Chu and Fischer, 1962; Evans and Mengel, 1964). It was proposed that CdR competed competitively with ara-C for the CdR kinase and thus prevented phosphorylation of the ara-C nucleoside.

The exact mechanism whereby the nucleotide derivatives of ara-C inhibited DNA synthesis remains as controversial still as it was when this author reviewed the problem in 1969 (Gibson, 1969). To begin with, it was proposed by Chu and Fischer (1962) that a possible mechanism for the inhibition of DNA synthesis by ara-C was a blockage of the precursors for DNA synthesis. The same authors suggested, as it was subsequently verified also by others, that the reduction of cytidine diphosphate (CDP) to deoxycytidine diphosphate (dCDP) (Chu and Fischer, 1962, 1965; Karon et al, 1966; Kimball et al, 1966) was prevented by a diphosphate derivative of ara-C (Chart 3). Thus, DNA synthesis rapidly came to a halt since the precursor $dCTP$ was essential for DNA synthesis in mammalian cells (Chu and Fischer, 1968b). However, observations by Moore and Cohen (1967) and Kaplan et al (1968) indicate that ara-C probably did not inhibit DNA synthesis by blocking the reduction of CDP. Graham and Whitmore (1970a) demonstrated that ara-C treated L-cells could still synthesize sufficient quantities of

dCDP nucleotides from exogenous deoxycytidine to maintain some DNA synthesis for several hours; yet, DNA synthesis was inhibited by 83%. Thus it appears that the inhibition of DNA synthesis by this particular mechanism may be doubtful. It should be noted further that in L5178Y cells, resistant to the inhibitory effects of ara-C, there was an augmentation of the pool size of dCDP over non-resistant strains (Momparler et al, 1968).

Another possibility conceivably explaining the mechanism of DNA synthesis inhibition by ara-C was the incorporation of a phosphorylated derivative of ara-C into the DNA and RNA molecules. Such incorporation of phosphorylated derivatives of ara-C into nuclei acid fractions by internucleotide linkages was reported first by Chu and Fischer (1965) in L5178Y cells. Incorporation of ara-C into DNA and RNA of replicating mouse fibroblasts (Silage, 1965) and human leukemic cells (Creasey et al, 1966) have been reported likewise. Momparler (1969) suggested that ara-C inhibited DNA synthesis by being incorporated at the end of the growing DNA chain, and thus blocked its elongation. Nonetheless, there appears to be much controversy in regards to the mode of incorporation of ara-C into DNA.

In contrast, Cardeilhac and Cohen (1964) observed that tritium-labeled arabinosylcytosine nucleotides were not incorporated during DNA synthesis. It was further suggested by Cleaver (1969) that certain compounds, such as actinomycin D or crystal violet known to bind with DNA, inhibited both ordinary and repair DNA replication. At the same time the same authors determined that ara-C did not interfere with repair DNA replication, and suggested therefore that ara-C did not bind or become incorporated into the DNA molecule. Moreover, Graham

and Whitmore (1970a) asserted that if incorporation was the cause of inhibition, then it should be lethal at all dosages, because DNA synthesis must resume in order for the cells to divide to form colonies. In another report (1970b) they present evidence that any incorporation of ara-C derivatives not confined to the terminal position would be unable to block any extension of DNA strands. It is thus evident that the question still remains as to whether ara-C becomes at all incorporated, and if so, how cells were able to recover to resume DNA synthesis and subsequent cell division.

A mechanism by which ara-C derivatives inhibit DNA synthesis most likely was the interference of DNA polymerase (Chart 3). Furth and Cohen (1968) stated that ara-CTP competively inhibited bovine lymphosarcoma and calf thymus DNA polymerase. Such an inhibition prevented the incorporation of ara-CTP into the DNA molecule. Similar inhibitory effects were reported also by Inagaki et al (1969) on the activity of crude DNA polymerase from both human leukemic leukocytes and Ehrlich ascites tumor cells. Recently, Graham and Whitmore (1970b) claimed that of all biochemical data so far available, the most probable mechanism of DNA synthesis inhibition by ara-C was a competetive inhibition of DNA polymerase by ara-CTP. Yet, there remains some information suggesting that there still may be an alternate mechanism whereby DNA synthesis becomes inhibited.

A group of workers reported that oral treatment of mice with ara-C was less effective on various tumors (Evans et al, 1964b; Dixon and Adamson, 1965; Kline et al, 1968) than was the parenteral (s.c., i.p., or i.v.) treatment. For instance, Skipper et al (1967) stated that after a single i.p. injection of a 50 mg/kg of ara-C dosage the

effective blood levels persisted for just 1-2 hours. Twenty-four hours after a parenteral administration of 20 mg/kg of H^3-ara-C in mice, 85% of the radioactivity was excreted (Dixon and Adamson, 1965). Similar observations were reported in the monkey (Mellett et al, 1971). Ara-C became very quickly deaminated by the kidney to uracil-arabinoside (ara-U), an inactive form, (Camiener and Smith, 1965; Saslaw et al, 1966). The absence of any antitumor effect after an oral administration was ascribed to the lack of absorption of the highly water soluable ara-C and any small quantity of ara-C that was absorbed by the intestine became quickly deaminated in the kidney. Interestingly enough different regimens in s.c. injections of ara-C (4x and 2x daily) enhanced the antileukemic effect over single daily doses (Kline et al, 1966). In contrast, different regimen in oral administration (Kline et al, 1968) did not affect the antileukemic properties of ara-C. Four daily oral doses of ara-C merely increased the toxicity with no increased antitumor effects.

Neil et al (1970) demonstrated that tetrahydrouridine (THU) enhanced the effectiveness of orally administered ara-C in leukemic (L1210) mice. THU inhibited the deaminase which converted ara-C to ara-U, thus effecting an increased plasma level of ara-C. Yet, the increased plasma levels of the ara-C-THU combinations was only 20% of those after i.p. injections; still, they were 3 to 5 times higher than when ara-C was solely administered by the oral route.

Intense oral ara-C therapy caused a definite retardation of growth of a human colonic neoplasm (GW-77) grown in the golden hamster (Goldenberg et al, 1968a). Persistent effects and levels of ara-C, as observed in the monkey (Mellett et al, 1971), were likewise noted

in the hamster studies. In fact, the inhibition of the growth rate of the human tumors in the hamsters was over 40% on the 9th day after the initiation of the therapy. Goldenberg et al, (1968b) succeeded to obtain a complete regression of a murine lymphatic leukemia (L1210/Mes) by a 6 day oral therapy schedule. In man, orale therapy of ara-C was followed by megaloblastic changes in bone marrow similar to those encountered after parenteral treatment of ara-C. This indicated that ara-C must have been resorbed by the intestine in sufficient quantities to produce these bone marrow changes (Goldenberg et al, 1968b).

Inasmuch as ara-C, by some mechanism, prevented the duplication of DNA in proliferating cell populations, a marked reduction of mitotic figures would be expected following ara-C treatment. Such a phenomenon has indeed been witnessed in HeLa S-3 (Kim and Eidinoff, 1965), human leukemic (Brewen, 1965) and human WI-28 cells (Heneed and Nichols, 1967). The mitotic rates of in vivo B16 melanoma and Ehrlich ascites tumor cells became reduced significantly by a single ara-C injection (Gibson, 1969; Bertalanffy and Gibson, 1971). Therefore, all the evidence points to the likelyhood that ara-C inhibited cells while in the S phase of the cell cycle. Cells in the G_1 phase were prevented from entering the S phase, while those cells in the S phase at the time of ara-C administration were either killed outright or at least delayed in that phase. The magnitude of such effects was related both to the dose and the duration of the ara-C administration (Karon and Shirakawa, 1970). Nonetheless, there remained a segment late in the S phase where DNA replication proceeded beyond a point, so that ara-C did not affect those cells in this latter portion of the S phase (Karon and Shirakawa, 1969; Bertalanffy and Gibson, 1971). Cells in the G_2 phase of the

cell cycle at the time ara-C was available, continued to mitosis and on into the G_1 phase (Karon and Shirakawa, 1969). The overall effect of ara-C upon the cell cycle (generation) time of L5178Y cells was a prolongation of the total time, namely from 10 hours in untreated to 23 hours in treated cell cultures (Chu and Fischer, 1968a). Similar observations were made with B16 melanoma where the total cell cycle duration became likewise prolonged by a single ara-C injection. However, the S phase itself was shortened from 7.5 hours in untreated to 6 hours in treated tumors (Bertalanffy and Gibson, 1971). The increase in the overall length of the cell cycle time was likely explained by the fact that in Don-C cells it was determined that ara-C interferes with the passage of cells from S to G_2 (Karon and Shirakawa, 1969). This decreased passage rate may also account for the observations by Bremerskov et al (1970) that G_2 cells appeared blocked before entering mitosis, as a slower transit time would produce on observation an increase in the number of G_2 cells in that particular stage. CdR prevented cytotoxicity of ara-C in cells of all stages of the cell cycle. Furthermore, CdR reversed the cytotoxic effects of ara-C on G_1 cells but did not effect such reversal of S phase cells (Young and Fischer, 1968).

The inhibitory effect of ara-C upon DNA synthesis produced an "unbalanced growth" syndrome analogous to thymidine-deficient growth of mammalian cells where one major compound such as DNA synthesis was inhibited while the formation of the other compounds such as RNA and proteins, was allowed to continue. Such a situation ensues in a diminished cell viability and a decreased mitotic activity (Kim and Eidinoff, 1965). This condition also leads to the formation of giant

cells (Tally and Vaitkevicius, 1963; Gibson, 1969; Bremerskov et al, 1970). The exact cause of cell death evoked by ara-C has not been fully ascertained. Chu and Fischer (1968a) believed it conceivable that the levels of inhibitory derivatives of cytosine arabinoside were not uniform among individual cells of nonsynchronized populations, and therefore individual cells even in the same phase of the cell cycle would not contain identical inhibitory amounts of ara-C. Thus, a cellular heterogeneity in concentration of inhibitory cytosine arabinoside would contribute to a constant fractional kill of cell population. Cells containing larger quantities of ara-C were killed and could not be rescued by CdR. In contrast, cells with lower concentrations could still be rescued by CdR (Chu and Fischer, 1968a,b). Nonetheless, it was shown by Lieberman et al (1970) that suppression of DNA synthesis alone may be enough to cause death in some proliferating tissues. These authors observed that if protein synthesis was simultaneously inhibited by cycloheximide or tenuazonic acid, along with DNA synthesis, cell death could be prevented in the intestinal crypts. These observations suggested that the synthesis of some protein(s) may play an important role in the lethal response associated with an interference with DNA synthesis. However, this protective effect of the protein inhibiting drugs (Lieberman et al, 1970) was selective; it was readily apparent in the epithelial cells of the intestinal crypt but was not so in lymphoid tissue. Karyorrhectic cells appeared both in lymphoid tissue and the intestinal epithelium after ara-C administration (Lenaz et al, 1969). Another possible mechanism for cell death by the incorporation of ara-C was observed in sRNA of cells. Chu (1970) reported that the increment of cell death correlated with

a linear increase of ara-C into the sRNA fraction of L5178Y cells in vitro.

In summary, it appears that one or more mechnisms may be responsible for acute cell death caused by ara-C. Graham and Whitmore (1970a) believed that any model for the ara-C killing of mammalian cells has to account for the following two conditions: (1) the inhibition of DNA synthesis and subsequent cell division for several hours was possible without affecting cell viability, and (2) why S phase cells are specifically killed in 1-2 hours after treatment with high concentrations of ara-C thus producing severe levels of DNA synthesis inhibition.

Other drugs have been employed experimentally in conjunction with ara-C, in an attempt to enhance the effectiveness of either drug in the regimen. Some of these compounds and their major advantages are listed in Table 1. The principal feature observed in most of the experiments listed was an increase in the mean survival time of the animals. This observation has been carried over even to the treatment of patients afflicted with various tumors.

Ara-C was employed clinically to combat a variety of leukemias (Carey and Ellison, 1965; Bodey et al, 1968; Burke et al, 1968; Howard et al, 1968; and Wang et al, 1971); carcinomas (Savel and Bruns, 1969; and Papac and Fischer, 1971); melanomas (Papac, 1968; and Savel and Burns, 1969); other solid tumors (Savel and Burns, 1969), and erythremia (Goldenberg, 1969). Generally speaking, ara-C produced substantial remissions in acute leukemia of children and adults, but also a few patients with solid tumors benefited. However, the remissions evoked by ara-C have been of brief duration unless they were followed up by

some other regimen of maintenance treatment. Maintenance therapy prolonged many remissions to be useful and comfortable with relatively little toxicity. The commonest adverse effects of ara-C treatment in children and adults were: leukopenia, thrombocytopenia, bone marrow suppression, nausea and vomiting.

Three reviews dealing with the clinical applications of cytosine arabinoside should be consulted for further information on this topic (Livingston and Carter, 1968; Frei et al, 1969 and an article by The Upjohn Company, 1970).

Table 1.

Combinations of ara-C and other drugs.

Explanation of Abbreviations:

ara-6-MP - 9-β-D-arabinofuranosyl-9-H-purine-6-thiol
BCNU - 1,3bis(2-chloroethyl)-1-nitrourea
CPA - cyclophosphamide
DIC - 5-(3,3-dimethyl-1-triazeno)-imidazole-4-carboxamide
5-FU - 5-fluorouracil
6-MP - 6 mercaptopurine
MTX - methotrexate
THU - tetrahydrouridine

References:

1. Burchenal and Dollinger, 1967
2. Evans et al, 1964a
3. Hoffman et al, 1969
4. Kimball et al, 1966
5. Kline et al, 1971
6. Mizuno and Humphrey, 1969
7. Neil et al, 1970
8. Roberts and Loehr, 1971
9. Tyrer et al, 1967
10. Vallamudi et al, 1968

TABLE 1

COMBINATIONS OF ARA-C AND OTHER DRUGS

Ara-C and	Cell Type	Therapeutic Effectiveness	Ref.
ara-6-MP	EAT	in vivo, produced an additive response	4
azaserine	EAT	in vivo, produced an additive response	4
BCNU	L1210	in vivo, prolonged survical time, additive response, greater effect on tumor cells than bone marrow	6,9
	sarcoma 180	in vivo, additive response	6
colcemid	L1210	in vivo, synchronized, prolonged survival time	10
CPA	L1210	in vivo, prolonged survival time	3
DIC	L1210	in vivo, prolonged survival time	5
5-FU	L1210	in vivo, prolonged survival time, did not increase antileukemic effect	1
mitomycin C	L5178Y L1210	in vivo, synergistin, prolonged survival effect	2
6-MP	L1210	in vivo, prolonged survival time, enhanced antileukemic effect	1
MTX	L1210	in vivo, prolonged survival time, did not increase antileukemic effect	1,5
	human lymphoblasts	in vitro, inhibits growth	8
porfiromycin	L5178Y L1210	in vivo, synergistic, prolonged survival time	2
THU	L1210	in vivo, inhibits deaminase that inactivates ara-C, thus increases effectiveness	7

CELL SYNCHRONY

Synchronized cell populations have been employed widely in biology to study events or responses dependent upon a particular position of the cell within the cell cycle. Synchronization enables the researcher to examine a particular event or response in cells of larger quantities than would be feasible without the synchronizing procedure. In fact, the production of a synchronized cell population produced much of the available information on the cell cycle. With few exceptions, cell synchrony was brought about chiefly *in vitro*, whereas limited work exists with *in vivo* cell populations. Hence, little is known at all about *in vivo* synchrony.

Before defining cell synchrony, an understanding of the asynchronous state is essential. A cell population, whether grown *in vitro* or *in vivo*, where cell division occurs randomly distributed in time is said to be *asynchronous*. That is, the mitoses are random because in their cycle-activities, cells are independent units (Zeuthen, 1964; Engelberg, 1964). Thus by definition, any deviation from the asynchronous state in a cell population signifies some degree of unbalanced growth and extrapolates towards the fully *synchronous* state. In contrast, a fully synchronous population is one where every constituent cell passes through a given point of the cell cycle at the identical point of time. There is some confusion in the literature between the words synchronous and synchronized. The word synchronous means that synchrony came about by natural selection, such as in tissue culture studies when all cells in mitosis were selected and grown as a synchronous culture, or else when synchrony arose as a natural occurrence, as for instance, in early

cleavage divisions of sea urchin eggs, or the synctium of slime mold (Nias and Fox, 1971). The metabolism of the cells is not altered in any way in synchronous growth even when the selection was carried out artificially (Scherbaum, 1964). Natural synchronous growth was not yet observed in any mammalian cell population (Nias and Fox, 1971). In contrast, the word synchronized implies that synchrony was produced artificially employing various chemical or physical agents, bringing about a temporal uniformity of all the cells with respect to one specific observable cellular event, such as mitosis or DNA synthesis for instance, such synchrony can be determined by H^3-thymidine. The production of a synchronized population, by the use of different means, likely disrupts the metabolic pattern(s) of the cell population as a whole, irrespective of the position of the cells in the life cycle (Scherbaum, 1964). Sinclair (1969) simply stated that synchronous implied unmodified, whereas synchronized meant modifications.

The methods of producing cell synchrony are multifold; still they can be classified under two general headings: physical and chemical methods. It should be kept in mind, however, that these techniques were primarily employed for producing synchrony in tissue culture.

The physical methods used are: mitotic, volume, gradient selection and temperature shock. The mitotic cell selection technique was introduced by Terasima and Tolmach (1961), taking advantage of the circumstance that monolayered cultured cells 'rounded up' during mitosis and could thus readily be removed from growing cultures. HeLa cell populations synchronized by this method attained a mitotic index of 90% (Terasima and Tolmach, 1963) and a H^3-thymidine labeling index of 90% (Petrovic and Nias, 1967). The percentage yield of this

mitotic cell selection method was quite low, however; various other methods were employed in an attempt to augment the yield of available cells. Among these were treatment with 0.06 μg/ml of colcemide to augment the number of mitotic figures; cooling the culture to 4°C for 1 hour to allow the dividing cells to loosen their attachment to the glass; and further a reduced calcium method which likewise decreased the affinity of mitotic cells to cling to the glass (Nias and Fox, 1971).

Another physical method for producing a synchronized cell population is by volume selection. Sinclair and Ross (1969), comparing the size distributions of asynchronous and synchronous populations with the Coulter electronic counter observed that the cells of synchronized populations exhibited an increased volume as a function of time throughout the generation cycle.

Gradient techniques were employed to some extent with non-attaching cells in cultures, such as mouse lymphomas which cannot be grown as monolayers (Nias and Fox, 1971). Comparatively, the results of this approach when compared to mitotic selection techniques, are quite poor.

Employing temperature shock, can produce a relatively good degree of cell synchrony (Newton, 1964). Yet, the exact mechanism responsible for such cell synchrony by this chilling method is not clear. Authors suggested that certain enzymes responsible for successive cell divisions were rapidly replaced, thus allowing cells to prepare for DNA synthesis and cell division more swiftly.

The chemical procedures for producing cell synchrony were: (1) block and release of cells at a particular phase in the cell cycle

and (2) killing cells while in a certain phase of the cell cycle. Perhaps the best blockage and release agents are those that inhibit cells in the DNA synthesis phase (Sinclair, 1969). Drugs that were observed to block DNA synthesis are amethopterin, FudR (5-fluorouradine), excess thymidine, hydroxyurea, and ara-C. All these drugs killed or arrested those cells that were engaged in DNA synthesis at the time of the application; they prevented thereby the cells in the other stages of the cycle (G_2, M and G_1) from entering the S phase. If the drug remains active during at least one generating time, a fair number of cells will accumulate at the end of G_1. When the drug is removed from the culture, the cells no longer inhibited will flow into the S phase as a single cohort. Thus, in general, satisfactory synchrony can be produced by these DNA inhibitors. However, one disadvantage is that moribund cells are also produced. A further drawback is that various metabolic properties of the synchronized cells become altered by the synchronizing drug.

Another group of blockage and release agents, acting at a different phase of the cycle, are vinblastine and colcemide. They block the cells in the M phase of the cell cycle. A fairly good degree of cell synchrony was achieved in some cell populations with these agents. Yet they also interfere with normal cellular processes.

A principal physico chemical method for producing cell synchrony is by the S-cell killing or suicide technique, developed by Whitmore and Gulyas (1966). It involves the uptake of high specific activity tritiated thymidine (HSA-^{3}HTdR) by cells in the S phase. The HSA-^{3}HTdR, administered in lethal quantities, kills cells in the S phase during a period greater than tC-tS. This allows a group of cells that were not

engaged in DNA synthesis to enter S after removal of the lethal amounts of HSA-^{3}HTdR. These cells entering S will become synchronized. A serious drawback of this technique, however, especially for those wishing to perform biochemical or cytological studies, is the large number of moribund cells (Sinclair, 1969).

The partial synchronization action of the specific DNA inhibitor, ara-C, was first reported after studies of cultured HeLa S-3 cells (Kim and Eidinoff, 1965); and Ehrlich ascites and B16 melanoma tumor cells grown in vivo (Bertalanffy and Gibson, 1971). Kim and Eidinoff (1965) stated that ara-C was able to block mitotic activity for a period of 22 hours. Eight hours after the ara-C was removed from the culture media, the mitotic index attained 300% of the control value. These authors determined further the synchronization index (F) to be 0.48 according to the method of Blumenthal and Zahler (1962); the latter authors asserted that an F value of 0.8 represented an excellent degree of synchrony. The generation time of the HeLa cell strain was 22 hours. Kim and Eidinoff (1965) assumed that a continuous effective level of ara-C (8.2×10^{-6}M) maintained for 22 hours allowed those cells in the late S, G_2, M, and G_1 phases to flow around the cell cycle and pile up before commencing the S phase. Any cells that did not reach that point in the cycle would not have been in the proliferative fraction of the cell cycle during the 22 hour block period. On removal of ara-C from the culture media, the cells halted at the end of G_1 would pass as a cohort into the S phase, through G_2 into mitosis. In fact, the cohort of cells attained mitosis 8 hours after the release of the synchronized cohort; hence, the duration of S and G_2 phases equalled 8 hours. Although the synchrony

produced in these experiments was low, it still indicated ara-C to be a potential synchronizing agent.

The production of cell synchrony in vitro evolved some synchrony criteria to judge the effectiveness of a particular synchrony technique; they serve also for comparing between observations of two or more synchrony experiments. Nonetheless, Sinclair (1969) asserted that it would be indeed difficult to conceive any single ideal criterion.

Criteria that evolved were usually confined to the first generation cycle immediately following effective synchronization, because the decay of any cell synchrony usually commences soon after the cessation of the synchrony procedure (Engleberg, 1964). Thus, continuous synchrony of several cell generations is unlikely to occur in mammalian cells (Nias and Fox, 1971). The reason for the rapid decay of synchrony in any cell population is ascribable to a number of factors inherent in that population. Those populations which have a wide range of cell cycle times (generation time) undergo decay of synchrony much more rapidly than cell populations where the generation times have a rather narrow range. Thus populations with a wide range of generation times quickly loose their synchrony and return to the asynchronous state. Kubitschek (1962) observed that the generation time of a cell population was simply an average distribution with a standard deviation (S.D.) of the range of 10-20%.

The various criteria employed to yield a measure of the degree of synchrony were based on the particular methods producing cell synchrony. They were: cell growth, cell sizing, cytological criteria, as well as identification of cells in DNA synthesis by H^3-thymidine incorporation (Sinclair, 1969). The first two criteria were discussed

by Dr. Sinclair (1969) and remain applicable solely to *in vitro* conditions of cell synchrony. In contrast, the second two criteria, namely the cytological criterion of mitotic index (MI) and the application of H^3-thymidine (labeling index) are applicable to *in vivo* cell synchrony as well.

Sinclair (Figure 8, p. 100, 1969) cited experiments where Chinese hamster cells were synchronized by selection. In these experiments, the percentage MI rose substantially above the MI of the asynchronous Chinese hamster cell population. In one synchronized population the MI reached 34% at 10.5 hours after synchronization, in the other merely 14.5% after the same interval. The MI of the asynchronous population remained steady at 7%. However, as Sinclair (1969) emphasized, the MI should be no more than a guide to the degree of cell synchrony achieved, as it does not define the state of the remaining cells that were not in mitosis at the time. There is a possibility of error because the nonmitotic cells may have approached closely to mitosis; thus synchrony can be quite good despite a low MI. That error is related partly to the comparitively short duration of mitosis compared to the length of the cell cycle, and hence, relatively few cells are in the mitotic phase at any given instant. The labeling index (LI) is not as vulnerable to this criticism as the duration of the S phase in relation to the entire cell cycle time is much greater (Nias and Fox, 1971).

The duration of the S phase during the cell cycle served as an indicator of synchrony (Sinclair and Morton, 1965) by determining the percentage of cells pulse-labeled (15 minutes) with H^3-thymidine at different times after synchronization.

A theoretical cell population is schematically presented in Chart 4. In a population where all cells are perfectly synchronized, the percentage of cells labeled should be zero until the end of G_1, 100% during the S phase, and zero again during G_2. The actual labeling index of synchrony can be determined by the equation (Sinclair and Morton, 1965):

$$L = L_{max} - L_{min}$$

where L = labeling index of synchrony

L_{max} = fraction of cells labeled at maximum of such a curve (Chart 4)

L_{min} = fraction of cells labeled at minimum (following the peak percentage of labeled cells).

The percentage of labeled cells in a theoretical asynchronous population is arbitrarily indicated by a straight line at about 20% (Chart 4). In a synchronized cell population the percentage of labeled cells forms a curve. Yet, the peak would not usually attain 100%, but fall short depending on the proportion of cells that did not become synchronized. In the theoretical synchronized population depicted by Chart 4, the L_{max} was arbitrarily placed at 94%, the subsequent low of the percentage labeled cell curve or L_{min} was 15%. The labeling index of synchrony, or L, can be determined from equation to be: 94% - 15% = 79%. A labeling index of synchrony of 79% indicates a satisfactory degree of cell synchrony of any cell population.

In vivo synchrony of mammalian cells is still in its infancy stage. At the time of writing, merely three laboratories supplied any information on in vivo synchrony. Lampkin et al, (1969) achieved a low degree of cell synchrony in acute leukemia of man after a single

Chart 4.

A theoretical plot of an asynchronous, synchronized and perfectly synchronized cell populations after a 15 minute pulse label of H^3-thymidine. Synchrony Index (SI) = 94% (L_{max}) - 15% (L_{min}) = 79%.

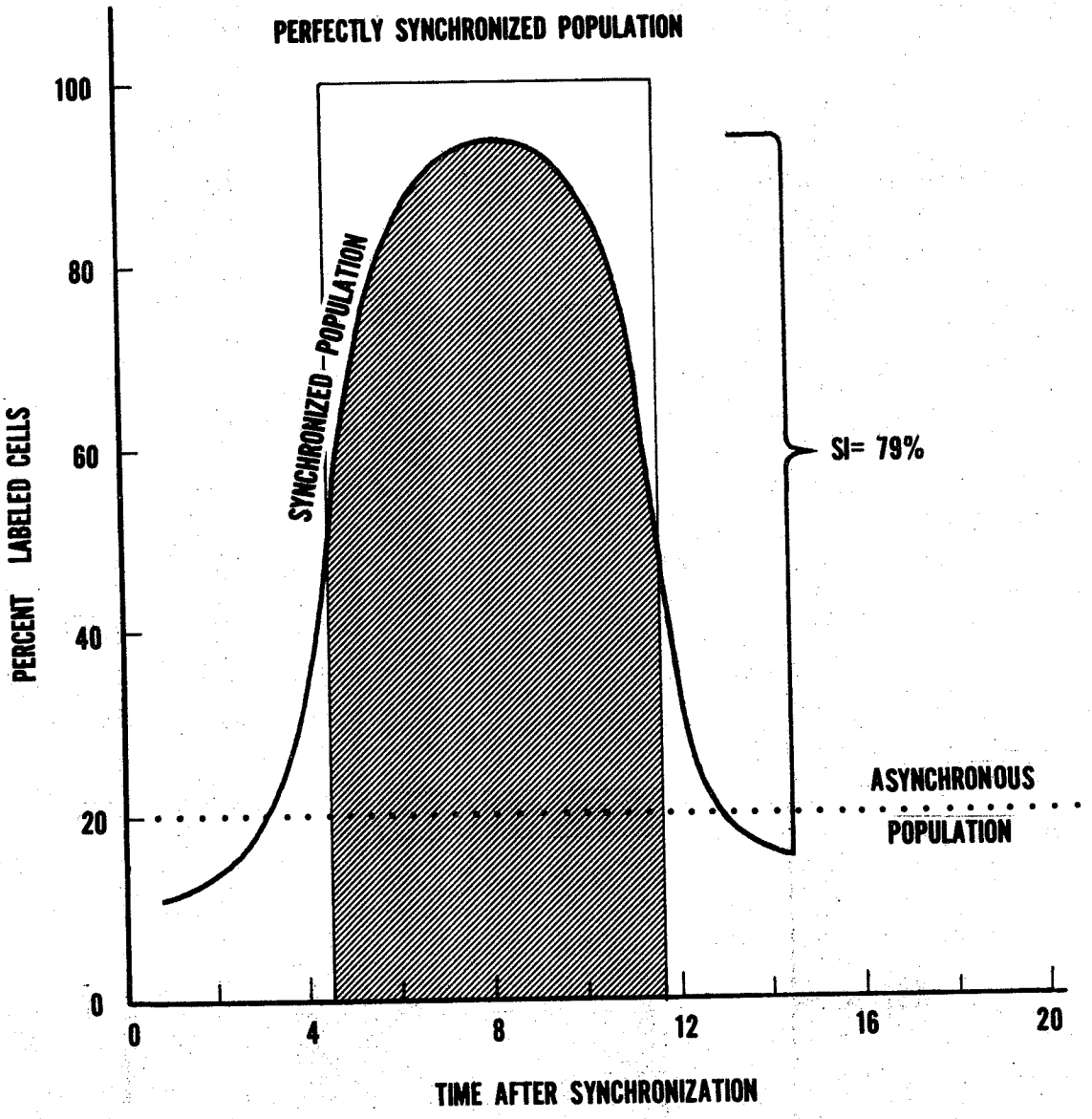

PERFECTLY SYNCHRONIZED POPULATION
100
80
60
40
20
0
PERCENT LABELED CELLS
SYNCHRONIZED-POPULATION
SI= 79%
ASYNCHRONOUS
POPULATION
0
4
8
12
16
20
TIME AFTER SYNCHRONIZATION

Chart 4

injection of ara-C. The percentage of labeled lymphoblasts declined immediately after the ara-C administration; by 48 hours the percentage had risen above the pretreatment level. Eighty-four hours later the percentage of labeled lymphoblasts was almost double that of the pretreatment level. After a peak of about 33%, the percentage of labeled lymphoblasts declined below the pretreatment level. This increase in the percentage of labeled cells signified that some degree of *in vivo* synchrony had occurred.

In the same year at Washington University School of Medicine, St. Louis, Missouri, Mauro and Madoc-Jones produced *in vivo* synchrony in murine lymphoma cells (Mauro and Madoc-Jones, 1969; Madoc-Jones and Mauro, 1970). Administering a single dosage of hydroxyurea (HU) these authors produced synchrony of the surviving lymphoma colony forming cells, as measured by the spleen-colony technique. The synchronization index attained was 0.56, as calculated by the procedure of Blumenthal and Zahler (1962).

Perhaps most informative is the report on *in vivo* synchronization by Rajewsky (1970). That author produced synchrony in a transplantable rat carcinoma by temporarily inhibiting DNA synthesis with hydroxyurea. The index of synchrony achieved was 0.5. This work by Rajewsky will be discussed more fully in the discussion section.

CHAPTER III

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

The host animal used in these experiments was the male C57BL/6J mouse obtained from the Jackson Laboratory, Bar Harbor, Maine. On arrival, the mice were 5-6 weeks, and they were employed in the experiments 1-2 weeks later; by that time they weighed 18-26 gms.

The mice were housed 4-6 animals per cage and were kept on a regulated 12 hour lighting schedule, with the dark period time to commence at 8:00 p.m. The mice were fed Purina chow and tap water ad libitum.

TUMOR MATERIAL

The tumor employed in all the experiments was the murine B16 melanoma (Figures 1-5), initially obtained in donor mice from the Jackson Laboratory, Bar Harbor, Maine. This tumor had once appeared spontaneously in the skin at the base of the ear of a C57BL/6J mouse in 1954 (Green, 1968). By December 1971, the tumor had undergone 465 transfers since 1954.

Tumor tissues were transfered to experimental mice from tumors which had been growing in the donor mice for 12-14 days. The donor animals were killed by cervical dislocation and the tumor was removed. It was placed in a petri dish containing Hank's balanced salt solution (pH 7.2-7.6) and minced into small pieces with scalpel blades. The experimental mice were lightly anaesthetized with ether, and a small cutaneous incision was placed in the lower right abdominal region. Through the incision a small pocket was made between the skin and the peritoneal wall with an alcohol cleaned probe. One or two small pieces of non-necrotic tissue were inserted in the cutaneous pocket

Figure 1.

Photomicrograph of B16 melanoma, showing it to be a relatively homogenous tumor cell population. In the centre of the field is a metaphase and a prophase, indicated by the arrows. In the right upper corner is a cross section of a capillary. H & E, x354.

Figures 2 & 3.

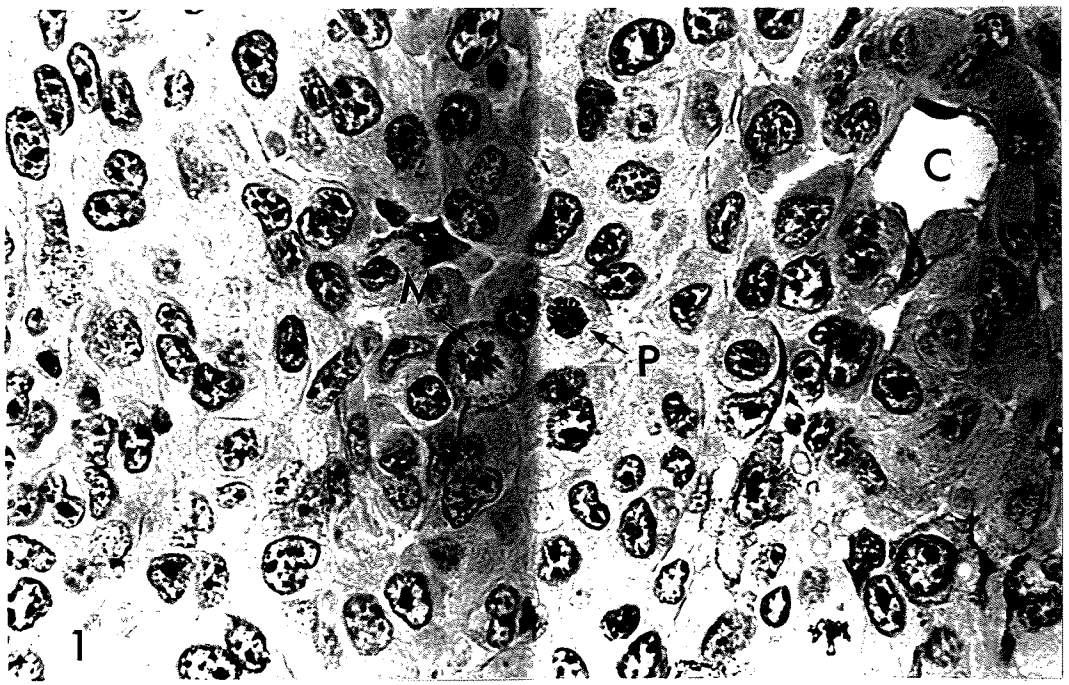
Photomicrographs of regions of B16 melanoma with degenerating cells, with shrunken nuclei and cytoplasm. H & E, x354.

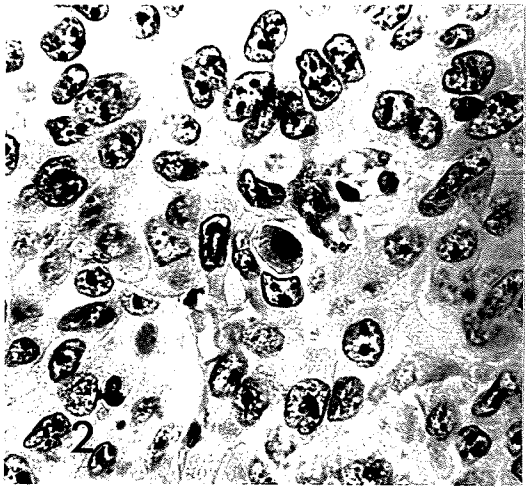
Figure 4.

Autoradiograph of B16 melanoma tumor cells after a pulse label of H^3-thymidine. At the right is a labeled metaphase. Both labeled and unlabeled interphase cells are visible. H & E, x881.

Figure 5.

Autoradiograph of B16 melanoma tumor cells. Both unpigmented and pigmented cells are present. The large cell in the centre is both pigmented and labeled with H^3-thymidine. The melanin occurs as dark granules, while the silver grains of the autoradiograph appear as hollow circles. H & E, x1052.


C
M
P
1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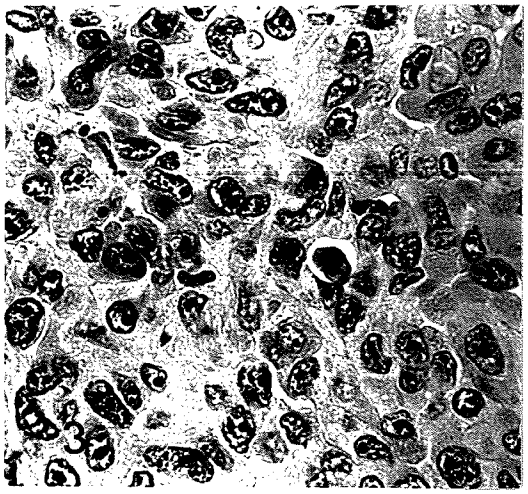
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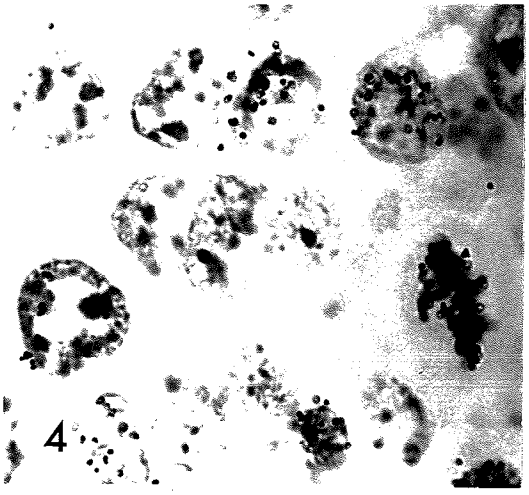
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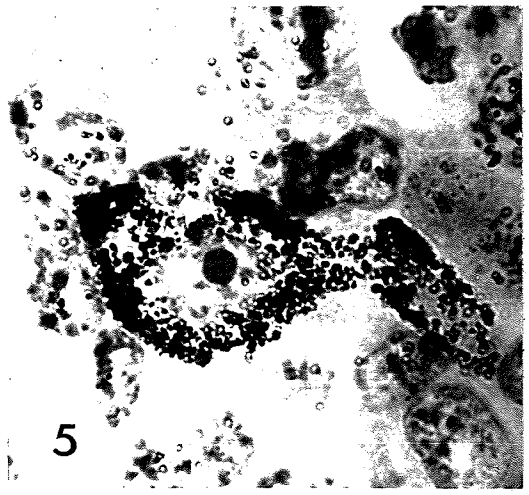
5

and the incision closed with a 11 mm. Michel wound clip.

The transfer of tumors was usually performed at 10 a.m. and the day designated as day 0 of tumor growth. Therefore, the first day of tumor growth began at 10 a.m. on the following day lasting for 24 hours to 10 a.m. again on the next day.

CYTOSINE ARABINOSIDE

Cytosine arabinoside (ara-C) was employed in the experiments to produce cell synchrony and ascertain the effects of a DNA synthesis inhibitor on the cytokinetics of a tumor cell population. Ara-C (U-19, 920: Lot nos. 8240-BDA-120 and 9580-BDA-50) was received complimentary from Cancer Research, Upjohn Company (Kalamazoo, Michigan).

The white crystaline powder of ara-C was readily soluble in distilled water, and if stored at refrigerated temperatures remained stable for approximately 7 days (Livingston and Carter, 1968). Yet, a fresh solution of ara-C in distilled water was prepared before each of the present experiments. Different concentrations of such ara-C solutions were administered by i.p. injections in 0.22 cc. volumes. These dosages were standardized by basing them on a mean body weight of 22 gms. of the tumor bearing mice (Gibson, 1969); they were expressed as mg of ara-C/kg body weight.

PREPARATION OF TISSUE SPECIMENS

The mice were killed by cervical dislocation. Two cubes of tumor tissue from peripheral non-necrotic areas were immediately excised and fixed in Davidson's fixative. Both tumor specimens were routinely processed and blocked in paraffin. One block served as a reserve, the other supplied 3-4μ thick microscope sections. Two microscope slides,

each containing 2-4 sections, were prepared from each tumor. Only every tenth section was placed on glass slides to prevent the scoring of the same nucleus twice in contiguous sections. One of the preparations was stained with hematoxylin and eosin, whereas the other, in experiments with H^3-thymidine, was prepared by the autoradiographic technique.

Tumor sections in which labeling indices were to be determined were diped in Kodak NTB^2 type liquid nuclear track emulsion and left to expose for 7 to 10 days in black boxes in the refrigerator. The sections were subsequently developed in Kodak D-19 developer, fixed, and stained with hematoxylin (Kopriwa, 1967; Gibson, 1969).

SCORING OF DATA

The enumeration of nuclei was performed with a binocular light microscope. To begin with, the sections were scanned under low magnification to locate an area of representative viable tumor tissue. Under oil immersion (1000 x), those nuclei contained in an area delimited by a micrometer disc (Bausch and Lomb) were counted. After those in the field were counted the slide was moved along one of the coordinates of the microscope stage and randomly stopped at some distance from the first field, in line with Mendelsohn's (1960) procedure; the nuclei in that field were ennumerated. This procedure was continued until a minimum of 1000 cells per tumor were scored. This evaluation of the specimens was performed without knowledge of the experimental nature of the particular samples. The cell scoring was carried out by one person. The nuclei of four tumors were scored thusly for each experimental group, amounting collectively to more

than 4000 cells.

KINETIC PARAMETERS AND THEIR DETERMINATION

The absolute growth curves of B16 melanoma were determined by measuring with calipers the greatest and least diameters of subcutaneous tumors of lightly anaesthetized mice at 1-day intervals from the time they became palpable. The diameters were converted to an approximation of mass by the formula (Simpson-Herren and Lloyd, 1970):

$$\frac{\text{length} \times (\text{width})^2}{2} = \text{mass (mg)}. \qquad (1)$$

The mass doubling time (MD_t) was that time required for the tumor to double its mass. The diameters were converted also to the area of a rectangle that enclosed the tumors, according to the formula (Steel et al, 1966):

$$\text{length} \times \text{width} = \text{area } (mm^2). \qquad (2)$$

Both the mean mass and area estimates were plotted on semi-log paper against time to produce growth curves.

The mean survival time of B16 melanoma bearing mice was obtained by recording the number of days between tumor transplantation and death of the animals.

The mitotic index (MI) of the tumors was determined by enumerating the number of mitotic cells present among 1000 tumor cells. The criteria accepted to identify the various stages of mitosis were those designated by Leblond and Stevens (1948). Prophase was identified by the appearance of definite chromatin filaments in the nucleus and

along its border. In some stages, the chromatin filaments had thickened, the nucleolus and nuclear membrane disappeared and the chromatin filaments spread throughout the cytoplasm. Metaphase was distinguished by an accumulation of the chromosomes at the equator, while anaphase was diagnosed by the separation of chromosomes which aggregated near the opposite poles of the cell. Telaphase began with the indication of a mid-plate dividing the cytoplasm, condensation of both nuclei, and terminated with the complete separation of two new daughter cells. The MI was expressed as a percentage figure.

The degenerating index (DI) of the tumors was ascertained by determinating the proportion of degenerating cells present among the viable tumor cells. Degenerating cells (Figures 2 and 3) were identified by dense shrunken nuclei, staining darkly blue with hematoxylin. The cytoplasm was likewise shrunken and scanty, and what remained stained brightly red with eosin. In contrast, the cytoplasm of nondegenerating B16 melanoma cells stained palely pink.

The DI, expressed as a percentage, was determined by the formula:

$$\frac{\text{degenerating cells}}{\text{nondegenerating cells + degenerating cells}} = \text{DI.} \qquad (3)$$

A total of at least 1000 cells was scored for each tumor. It has to be noted that degenerating cells were omitted from all other indices determined in these studies.

The percentage of colchicine metaphases for the 2 hour period was expressed as the 2 hour mitotic rate (MR) of the B16 melanoma. The colchicine (Inland Alkaloid Inc., Tipton, Indiana) was injected i.p. in a dosage of 0.2 mg per 100 gm body weight. The percentage of

colchicine arrested metaphases was determined on different days of tumor growth. To account for any lag of colchicine action, the percentage equivalent to half an hour was subtracted from the percentage of colchicine metaphases occurring 2½ hours after the administration of the drug. The ½ hour interval was accepted because this lag period of colchicine action was observed previously in other cell populations (Bertalanffy et al, 1965; Clark, 1970). Accordingly, the percentage of colchicine metaphases was corrected for the B16 melanoma cell community. At least 1000 cells were counted per tumor specimen; the number of colchicine metaphases were separately recorded. Prophases were included with the "resting" cell category. Colchicine metaphases were recognized by the following features although not all were necessarily occurring simultaneously: (1) darkly staining clumps of chromatin usually in the center of the cell; (2) a peripheral halo of cytoplasm; (3) an enlarged cell; and (4) absence of a nuclear membrane (Wallace, 1964).

The daily mitotic rate (dMR) was estimated by calculating the percentage of cells entering mitosis during a 24 hour period, according to the formula:

$$\frac{\text{MR} \times 24 \text{ hours}}{\substack{2 \text{ hours} \\ \text{(interval of MR)}}} = \text{dMR}. \qquad (4)$$

From these data the doubling time (Dt), that is, the number of days required for the division of 100% of the cells, was determined by the formula:

$$\frac{100}{\text{dMR}} \times 24 \text{ hours} = \text{Dt}. \qquad (5)$$

The duration of mitosis (t_M), the time required for a cell to enter morphological prophase until it separates into two daughter cells, was estimated by the formula (Leblond and Stevens, 1948):

$$\frac{x \text{ MI}}{\text{MR for } x \text{ hours}} = t_M \text{ (hours)}. \qquad (6)$$

Tritiated thymidine (H^3-thymidine). This label specific for DNA was employed in the determination of the different kinetic parameters of B16 melanoma in combination with autoradiography (Figures 4 and 5). The thymidine preparation was obtained from Amersham/Searle, Don Mills, Ontario. H^3-thymidine (specific activity of 5 Ci/mM) was administered i.p. in a dosage of 25μC/0.25 ml. in distilled water to ascertain all H^3-thymidine parameters, apart from the cell cycle determination where 50μC/0.25 ml. in distilled water was employed. Once a cell had incorporated H^3-thymidine while engaged in DNA synthesis, it can be visualized by autoradiography (Hughes et al, 1958). It was determined at the outset in labeled cell smears that 2 grains per nucleus represented the maximum background. Consequently, any nucleus overlaid with more than 3 grains could be considered labeled.

The labeling (thymidine or radioactive) index (LI), was that percentage of cells that were labeled in a particular cell population, shortly after a single pulse label of H^3-thymidine (Baserga and Kisieleski, 1962). In the present report such data are expressed by LI, followed by the time designation, for instance, LI_{15} represented that percentage of B16 melanoma cells labeled 15 minutes after a pulse label of H^3-thymidine, or LI_{60} those cells labeled after 60 minutes. The LI data were determined by scoring at least 1000 cells per tumor.

The duration of the different phases of the cell cycle both in untreated and ara-C treated tumors, were determined from the percent labeled mitoses (PLM) curves. The cell cycle time (t_C) was that period between the midpoint of the ascent of the first peak and the midpoint of the ascent of the second peak of the curve. The duration of the S phase (t_S) was measured from the midpoint of the first ascent to the midpoint of the first descent of the curve. The duration of $G_2 + \frac{1}{2}M$ was the period between the injection of H^3-thymidine and the midpoint of the first ascent. The duration of mitosis (t_M) was determined by formula (6). The length of G_1 (t_{G1}) was equal to $t_C - (t_S + t_{G2} + t_M)$.

The growth fraction (GF) of a given population is represented by the proportion of proliferating (or growing cells) within a cell population. In this study, the GF of B16 melanoma was calculated by the formula of Mendelsohn (1962):

$$\frac{\frac{\text{labeled cells}}{\text{100 cells}}}{\frac{\text{labeled mitoses}}{\text{100 mitoses}}} = \text{GF.} \qquad (7)$$

The regimen of ara-C administrations used to produce cell synchrony varied in dosage, number of injections and time intervals between the latter. They were as follows:

Series 1:	12.5 mg/kg	-	4 injections	-	4 hour intervals
Series 2:	25.0 mg/kg	-	4 injections	-	4 hour intervals
Series 3:	12.5 mg/kg	-	8 injections	-	1 hour intervals
Series 4:	12.5 mg/kg	-	8 injections	-	2 hour intervals
Series 5:	18.8 mg/kg	-	8 injections	-	2 hour intervals
Series 6:	12.5 mg/kg	-	16 injections	-	1 hour intervals
Series 7:	18.8 mg/kg	-	16 injections	-	1 hour intervals

The degree of synchrony produced by various regimens of ara-C injections was determined by the formula of Sinclair and Morton (1965):

The duration of the different phases of the cell cycle both in untreated and ara-C treated tumors, were determined from the percent labeled mitoses (PLM) curves. The cell cycle time (t_C) was that period between the midpoint of the ascent of the first peak and the midpoint of the ascent of the second peak of the curve. The duration of the S phase (t_S) was measured from the midpoint of the first ascent to the midpoint of the first descent of the curve. The duration of $G_2 + \frac{1}{2}M$ was the period between the injection of H^3-thymidine and the midpoint of the first ascent. The duration of mitosis (t_M) was determined by formula (6). The length of G_1 (t_{G1}) was equal to $t_C - (t_S + t_{G2} + t_M)$.

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Series 4:	12.5 mg/kg	-	8 injections	-	2 hour intervals
Series 5:	18.8 mg/kg	-	8 injections	-	2 hour intervals
Series 6:	12.5 mg/kg	-	16 injections	-	1 hour intervals
Series 7:	18.8 mg/kg	-	16 injections	-	1 hour intervals

The degree of synchrony produced by various regimens of ara-C injections was determined by the formula of Sinclair and Morton (1965):

$$L_{max} - L_{min} = L. \qquad (8)$$

where L_{max} = fraction of cells labeled (LI_{15}) at maximum following a synchrony procedure.

L_{min} = fraction of cells labeled (LI_{15}) at minimum (following the peak percentage of labeled cells).

L = labeling index of synchrony.

In this work L was called the synchrony index (SI) so that formula (8) would appear as:

$$L_{max} - L_{min} = SI. \qquad (9)$$

STATISTICS USED

The factorial analysis of variance (MCST 12) and the standard "student - t" test served as measures of variance. The "student - t" test was used to compare means, in which $n_1 = n_2$ (Gray, 1961). A 5% level of significance either accepted or rejected the nul hypothesis.

A polynomial regression analysis was employed to compute the growth data to a best fit curve.

CHAPTER IV

RESULTS

The principal objectives of this investigation were to determine the growth characteristics of B16 melanoma, the production of in vivo cell synchrony in the same tumor employing ara-C a specific DNA inhibitor and the effects of ara-C upon the kinetics of both asynchronous and synchronized tumor cells.

The observations in this study are divided in the following three sections: (1) the normal kinetics of B16 melanoma on different days of tumor growth; (2) the production of cell synchrony by varying the dosage of ara-C, the number of injections and the intervals between the multiple administrations of the drug; and (3) effects of ara-C on the cell cycle, and cell kinetics of asynchronous and synchronized B16 melanoma cell populations.

Section I

This section deals with the kinetics of B16 melanoma on different days of tumor growth.

The growth curves of B16 melanoma were determined by superficial caliper measurements. The B16 melanoma became palpable on days 4-6 of tumor growth. The tumor diameters of several mice were measured daily. The number of tumor bearing mice was variable because some of the animals served simultaneously for other experiments or else as donor animals, and therefore were killed at different intervals. Other animals served these measurements daily until they succumbed to the tumor. A summary of these measurements is tabulated in Table 2 and plotted on semi-log paper (Chart 5). It is evident that the tumors increased both in mean area and mass between day 4 to day 33 of tumor gorwth. The thirty-four tumors measured had a mean area of 21 ± 8 mm^2 and the mean mass was 45 ± 31 mg on day 4 of tumor growth.

TABLE 2

SUMMARY OF B16 MELANOMA GROWTH DATA

Days Post Implant of Tumor	Number of Tumors Measured	Area (mm^2) (mean ± S.D.)	Mass (mg) (mean ± S.D.)	Mass Doubling Time (hours)
4	34	21 ± 8	45 ± 31	
5	41	32 ± 10	79 ± 36	
6	43	35 ± 7	91 ± 38	48
7	43	38 ± 11	105 ± 39	
8	43	51 ± 20	162 ± 92	
9	43	75 ± 27	302 ± 194	
10	38	80 ± 37	324 ± 212	72
11	26	100 ± 53	462 ± 317	
12	26	138 ± 68	737 ± 521	
13	26	179 ± 74	1079 ± 718	
14	26	195 ± 109	1228 ± 704	
15	22	214 ± 80	1416 ± 755	
16	22	225 ± 60	1470 ± 795	
17	22	259 ± 79	1848 ± 848	
18	21	284 ± 85	2201 ± 1019	120
19	19	297 ± 89	2294 ± 981	
20	17	317 ± 103	2655 ± 1279	
21	16	306 ± 94	2412 ± 1093	
22	13	353 ± 96	2964 ± 1203	
23	13	386 ± 92	3346 ± 1052	
24	12	433 ± 106	4058 ± 1623	
25	11	439 ± 115	4412 ± 1533	168
26	10	525 ± 102	5496 ± 1671	
27	7	551 ± 171	6136 ± 2169	
28	6	556 ± 198	6322 ± 3085	
29	6	605 ± 167	7120 ± 3550	
30	5	572 ± 101	6132 ± 1586	
31	5	581 ± 104	6310 ± 1736	
32	3	668 ± 195	8277 ± 3769	
33	3	704 ± 136	8840 ± 2625	192

Chart 5.

Growth curves of B16 melanoma plotted from caliper measurements of the tumors at various stages of growth. Each animal bore a single tumor. The individual points of the curves represent the mean of 3-43 tumors measured at daily intervals post-implantation. The solid lines constitute the best computed fit to the mean values.

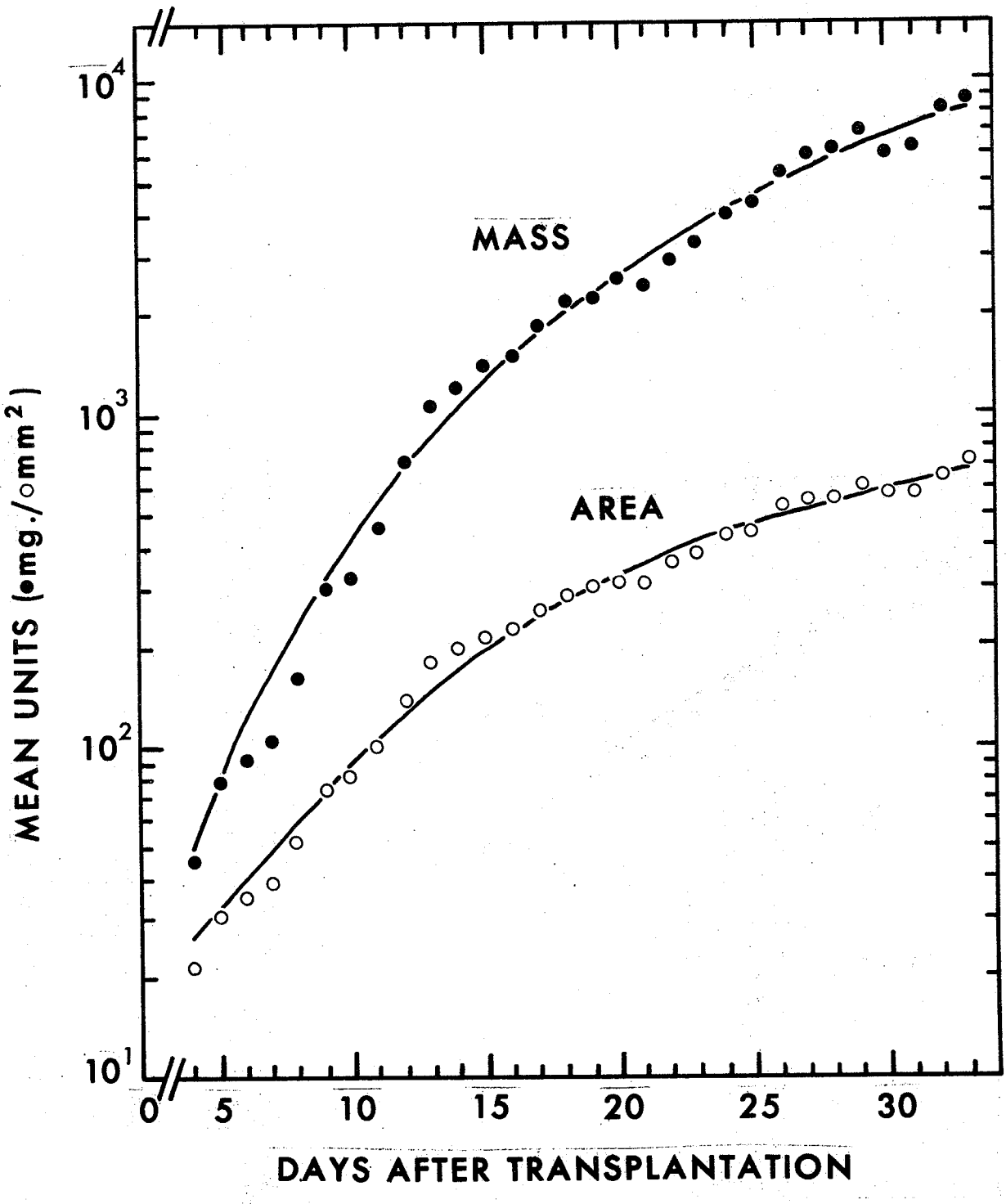

MASS
AREA
MEAN UNITS (•mg./○mm²)
10⁴
10³
10²
10¹
0
5
10
15
20
25
30
DAYS AFTER TRANSPLANTATION

Chart 5

On the 33rd day of tumor growth, only 3 tumors were measured, and the mean area was 704 ± 136 mm^2, the mean mass 8840 ± 2625 mg. The mass doubling time of B16 melanoma was approximately 48 hours on day 6 of tumor growth, 72 hours on day 10, 120 hours on day 18; it became further prolonged to 168 hours on day 25 and 192 hours on day 33 of tumor growth (Table 2).

In Chart 5, both the mean mass and area on different days of tumor growth are plotted. The best fit curve was determined for both sets of data by a polynomial regression analysis. The correlation coefficient for the mass data curve was 0.980 and 0.989 for the area data curve.

The mean survival time of C57BL/6J mice bearing B16 melanoma was calculated by averaging the life span of 109 tumor bearing mice. A histogram of this data is seen in Chart 6. The survival time of tumor bearing mice ranged from 15-46 days after transplantation. The histogram indicates a bimodal trend peaking at day 18 and day 31 of tumor growth. The mean survival time was 29.0 ± 6.9 days.

The mitotic rate and doubling time of B16 melanoma cell population were determined by the colchicine technique on different days of tumor growth. Twenty-four tumor bearing mice served as experimental animals. On day 6 of tumor growth, eight tumor bearing mice were administered colchicine i.p., and four of them were killed ½ hour after the colchicine injection, the remaining four, 2½ hours later. The identical procedure was applied to groups of mice on days 10 and 18 of tumor growth. The percentage of colchicine metaphases for each group, on days 6, 10 and 18 of tumor growth, are listed in

Chart 6.

A histogram of the survival times of C57BL/6J mice bearing B16 melanoma.

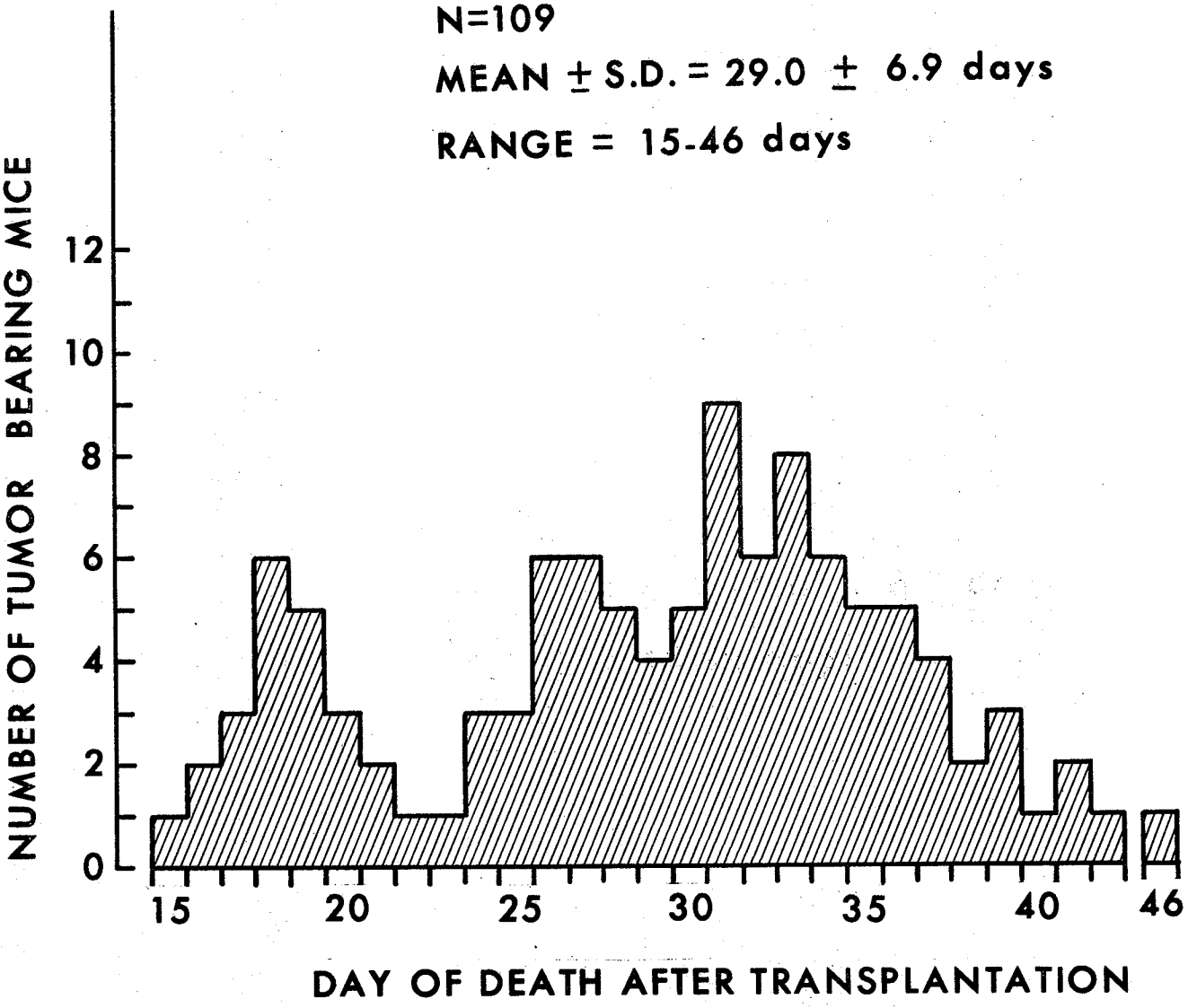

N=109
MEAN ± S.D. = 29.0 ± 6.9 days
RANGE = 15-46 days
NUMBER OF TUMOR BEARING MICE
0
2
4
6
8
10
12
15
20
25
30
35
40
46
DAY OF DEATH AFTER TRANSPLANTATION

Chart 6

in Table 3. The two hour mitotic rate of B16 melanoma was determined for days 6, 10 and 18 of tumor growth by subtracting the percentage of colchicine metaphases of the ½ hour group from the 2½ hour group. The mitotic rate on day 6 of tumor growth was 7.25; it declined to 4.69 and 1.71 on days 10 and 18, respectively. The doubling times of B16 melanoma on day 6, 10 and 18 were 27.6, 42.6 and 117.0 hours, respectively.

The effect of H^3-thymidine upon the mitotic and degenerating index of B16 melanoma, was determined with 16 tumor bearing mice. At 10 a.m. on day 10 of tumor growth, a group of four untreated tumor bearing mice, serving as controls, was killed. A second group of 4 mice received a single 15 minute pulse label of H^3-thymidine, a third group, a single 60 minute pulse label of H^3-thymidine. The fourth group of tumor bearing mice received 16 injections of H^3-thymidine at 1 hour intervals; they were killed 1 hour after the last H^3-thymidine injection. The mean percentages of labeled cells (LI), as well as the mitotic and degenerating indices determined by the above mentioned four groups are listed in Table 4.

The LI of that group of tumors receiving H^3-thymidine 15 minutes before being killed, was 20.20%; whereas after a 60 minute pulse label of H^3-thymidine it was 42.04%. When 16 injections of H^3-thymidine were administered at 1 hour intervals the LI of the tumor was 74.24%. Interestingly enough, the MI and DI of these three H^3-thymidine treated groups were statistically the same ($p > 0.05$). Neither were they dissimilar ($p > 0.05$) from the control group of animals not given H^3-thymidine (Table 4).

TABLE 3

MITOTIC RATE AND DOUBLING TIME OF B16 MELANOMA ON DAYS 6, 10 AND 18 OF TUMOR GROWTH

Day of Tumor Growth	Percent Colchicine Metaphases		Mitotic[a] Rate (2 hours)	Doubling Time (hours)
	½ hour after colchicine injection (mean ± S.D.)	2½ hours after colchicine injection (mean ± S.D.)		
6	2.97 ± 0.26	10.22 ± 0.75	7.25	27.6
10	2.07 ± 0.18	6.76 ± 3.44	4.69	42.6
18	1.22 ± 0.30	2.93 ± 0.50	1.71	117.0

[a]determined by subtracting the ½ hour column from the 2½ hour column.

TABLE 4

INDICES[a] OF B16 MELANOMA AT DIFFERENT TIME INTERVALS AFTER H^3-THYMIDINE ADMINISTRATION

Hours After H^3-thymidine Injection	Number of Injections	Labeled Cells (mean ± S.D.)	Mitotic Index (mean ± S.D.)	Degenerating Index (mean ± S.D.)
0	0	-	2.11 ± 0.26	0.97 ± 0.45
¼	1	20.20 ± 2.02	2.47 ± 0.49	1.05 ± 0.79
1	1	42.04 ± 4.78	2.53 ± 0.30	0.77 ± 0.17
1	16[b]	74.24 ± 7.99	1.92 ± 0.63	0.78 ± 0.20

[a] expressed as percentages.

[b] animals received 16 i.p. injections of H^3-thymidine at 1 hour intervals and were killed 1 hour after the last injection.

The mitotic, degenerating and labeling indices of B16 melanoma on days 6, 10 and 18 of tumor growth were determined. Twelve tumor bearing mice were divided into 3 groups of 4 mice each. The first group received a single i.p. injection of H^3-thymidine at 10 a.m. on day 6 of tumor growth and was killed 1 hour later. Similarly, the second and third groups of mice received a single i.p. injection of H^3-thymidine at 10 a.m. on days 10 and 18 of tumor growth, respectively, and also killed 1 hour later. By scoring autoradiographs, the LI_{15} was determined for each group, and listed in Table 5. Also listed in that table are the data of MI and DI for each of the three groups, determined in hematoxylin and eosin sections.

Both the MI and LI_{15} of B16 melanoma declined with tumor aging. The MI was 3.25% on day 6, 2.53% on day 10, and 1.32% on day 18 of growth. The MI values of the day 6 and day 18 tumors differed significantly ($p < 0.05$), whereas the MI of the tumor on day 10 did not differ either from those of the 6 or 18 day tumors ($p > 0.05$). The LI_{15} of day 6, 10 and 18 tumors were 47.74, 45.98 and 28.10%, respectively. The difference was not significant between the LI_{15} of the tumor on day 6 and 10 ($p > 0.05$). However, the LI_{15} of the day 18 tumors differed significantly from that of the LI_{15} of both 6 and 10 day tumors ($p < 0.05$). The DI of the tumors on the three days of tumor growth did not differ statistically ($p > 0.05$).

The growth fraction of B16 melanoma was determined by H^3-thymidine autoradiography. A single i.p. dosage of H^3-thymidine was administered to 28 B16 melanoma bearing mice at 10 a.m. on day 3 of tumor growth. The first group of four mice was killed four days later,

TABLE 5

MITOTIC, DEGENERATING AND LABELING INDICES OF B16 MELANOMA ON DAYS 6, 10 AND 18 OF TUMOR GROWTH

Day of Tumor Growth	Mitotic Index (mean ± S.D.)	Degenerating Index (mean ± S.D.)	Labeling Index (mean ± S.D.)
6	3.25 ± 0.36	0.75 ± 0.22	47.74 ± 5.01
10	2.53 ± 0.84	0.77 ± 0.48	45.98 ± 3.47
18	1.32 ± 0.31	0.83 ± 0.20	28.10 ± 7.34

that is on day 7 of tumor growth after having received the tracer. However, at necropy it became evident that two of the mice had not developed tumors. Therefore, only two tumors could be analyzed. The second, third and fourth groups of mice were killed on the 5th, 6th and 7th day after the single administration of H^3-thymidine, that is, on days 8, 9 and 10 of tumor growth. The fifth, sixth and seventh groups were killed 9, 11 and 13 days after the tracer was administered, corresponding to days 12, 14 and 16 of tumor growth. The percentages of labeled and mitotic cells were ascertained, and listed in Table 6. The growth fraction is likewise included in Table 6, and graphically presented in Chart 7. The percentage of labeled cells declined from 16.32% on the fourth day after the H^3-thymidine administration to 4.65% on the thirteenth day. The percentage of labeled mitoses likewise declines from 28.5% to 8.5% during the identical period. The GF ranged from 0.47 to 0.59 between days 7 to 16 of tumor growth. The mean value of the growth fraction was 0.53. A significant difference did not exist between the means of the GF on any of these days ($p > 0.05$).

The mitotic and degenerating indices were likewise determined from routine sections of the tumor (Table 7). The MI ranged from 2.04% to 2.72% between day 7 to day 16 of tumor growth. Whereas the DI ranged from 0.79% to 1.51%. Neither the MI nor the DI differed statistically ($p > 0.05$) during the 9 day span of the experiment.

The <u>proportion of B16 melanoma cells labeled after repeated H^3-thymidine administrations</u> in day 10 tumors.

Twenty-four tumor bearing mice were divided into 6 groups of 4 mice. H^3-thymidine was injected at 6 hour intervals beginning at

TABLE 6

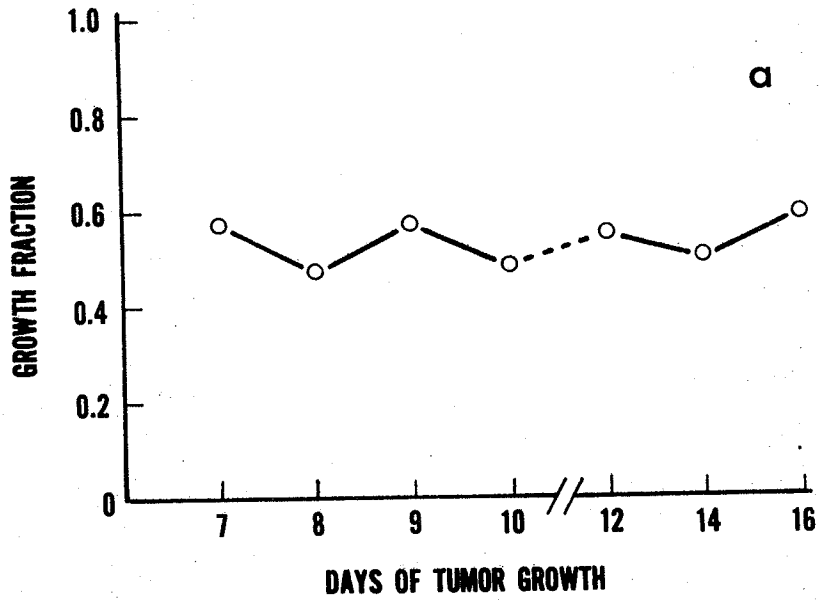
GROWTH FRACTION OF B16 MELANOMA ON DIFFERENT DAYS AFTER H^3-THYMIDINE ADMINISTRATION AND TUMOR GROWTH

Days After H^3-Thymidine/ Days Of Tumor Growth	Number of Tumors	Labeled Cells/ 100 Cells (mean ± S.D.)	Labeled Mitoses/ 100 Mitoses (mean ± S.D.)	Growth[a] Fraction (mean ± S.D.)
4/7	2	16.32 ± 2.66	28.5 ± 2.45	0.57 ± 0.00
5/8	4	11.66 ± 2.76	24.7 ± 3.40	0.47 ± 0.00
6/9	4	8.25 ± 2.52	15.0 ± 6.25	0.57 ± 0.10
7/10	4	7.39 ± 4.43	15.0 ± 4.47	0.49 ± 0.00
9/12	4	5.81 ± 1.59	10.8 ± 2.65	0.55 ± 0.10
11/14	3	4.29 ± 0.04	8.7 ± 1.00	0.50 ± 0.00
13/16	4	4.65 ± 0.74	8.5 ± 2.65	0.59 ± 0.45

[a]determined by the ratio of column 3 over column 4.

Chart 7.

Growth fraction of B16 melanoma. (a) The GF on different days of tumor growth. (b) Component indices of the GF. The GF_9 (○) and GF_{10} (●) for any particular point is the slope of the line joining the point of origin. Representative lines for the mean GF_9, GF_{10} and a GF of 0.3 and 1.0 were inserted.


a
GROWTH FRACTION
1.0
0.8
0.6
0.4
0.2
0
7
8
9
10
12
14
16
DAYS OF TUMOR GROWTH

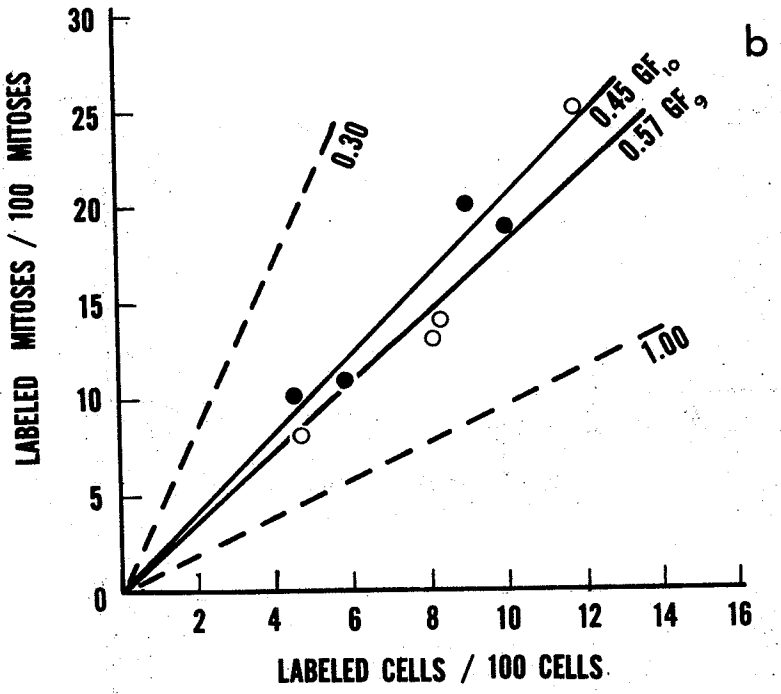

b
LABELED MITOSES / 100 MITOSES
30
25
20
15
10
5
0
2
4
6
8
10
12
14
16
LABELED CELLS / 100 CELLS
0.30
0.45 GF10
0.57 GF9
1.00

Chart 7

TABLE 7

MITOTIC AND DEGENERATING INDICES[a] OF B16 MELANOMA ON DIFFERENT DAYS OF TUMOR GROWTH

Day of Tumor Growth	Number of Tumors	Mitotic Index (mean ± S.D.)	Degenerating Index (mean ± S.D.)
7	2	2.63 ± 0.66	0.79 ± 1.26
8	4	2.41 ± 0.67	1.08 ± 0.52
9	4	2.72 ± 0.57	1.22 ± 0.51
10	4	2.09 ± 0.66	1.04 ± 0.62
12	4	2.04 ± 0.69	0.92 ± 0.73
14	3	2.56 ± 1.17	1.51 ± 0.55
16	4	2.33 ± 0.92	1.43 ± 1.04

[a]expressed as percentages.

10 a.m. The first group was killed at 10 p.m. after receiving 2 injections of H^3-thymidine at 10 a.m. and 4 p.m. or after 12 hours of repeated labeling. The second group was killed after 4 injections of H^3-thymidine or after 24 hours of repeated labeling. The third, fourth, fifth and sixth groups were killed after 36, 48, 60 and 72 hours of repeated labeling, respectively. The percentages of labeled cells are listed in Table 8, and graphically illustrated in Chart 8. The percentage of labeled cells increased steadily until it attained 89.06%, 72 hours after the first H^3-thymidine administration. The LI never reached the 100% level during the experimental period, however.

The duration of the phases of the cell cycle of B16 melanoma was determined on days 6 and 18 of tumor growth by percent labeled mitoses curves. To ascertain such data on the sixth day of tumor growth, 51 tumor bearing mice received a single i.p. injection of H^3-thymidine at 10 a.m. The animals were subsequently killed in groups of 3-4 mice at 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25 and 27 hour intervals after the H^3-thymidine administration. The percentage of labeled mitoses was determined in autoradiographs for each tumor. These values together with the group means are listed in Table 9 and graphically presented in Chart 9A. The cell cycle time (t_C) of B16 melanoma cells on day 6 of tumor growth was estimated from these data to be 12.5 hours; the duration of the S phase was 9.5 hours. The interval of the premitotic gap (G_2) and one-half of the duration of mitosis ($\frac{1}{2}M$) was calculated to last 1.5 hours. The duration of mitosis (t_M) was ascertained to be 0.9 hours. From these data, the length of the post-mitotic gap (G_1) and one-half of

TABLE 8

PROPORTION OF CELLS LABELED AFTER REPEATED INJECTIONS OF H^3-THYMIDINE IN 10-DAY TUMORS

Group	Number of Tumors	Hours of Repeated Labeling	Number of[a] H^3-Thymidine Injections	Labeled Cells (mean ± S.D.)
1[b]	4	¼	1	20.20 ± 2.02
2[b]	4	1	1	42.04 ± 4.78
3	4	12	2	55.72 ± 4.33
4	4	24	4	67.71 ± 9.99
5	4	36	6	78.23 ± 6.11
6	4	48	8	76.01 ± 5.26
7	4	60	10	83.36 ± 5.83
8	4	72	12	89.06 ± 3.28

[a] H^3-thymidine was injected i.p. every six hours.

[b] from Table 4.

Chart 8.

Proportion of cells labeled after repeated 6-hourly injections of H^3-thymidine in 10-day old tumors. Each point represents the mean percentage of tumors from 2-4 mice with the standard deviation.

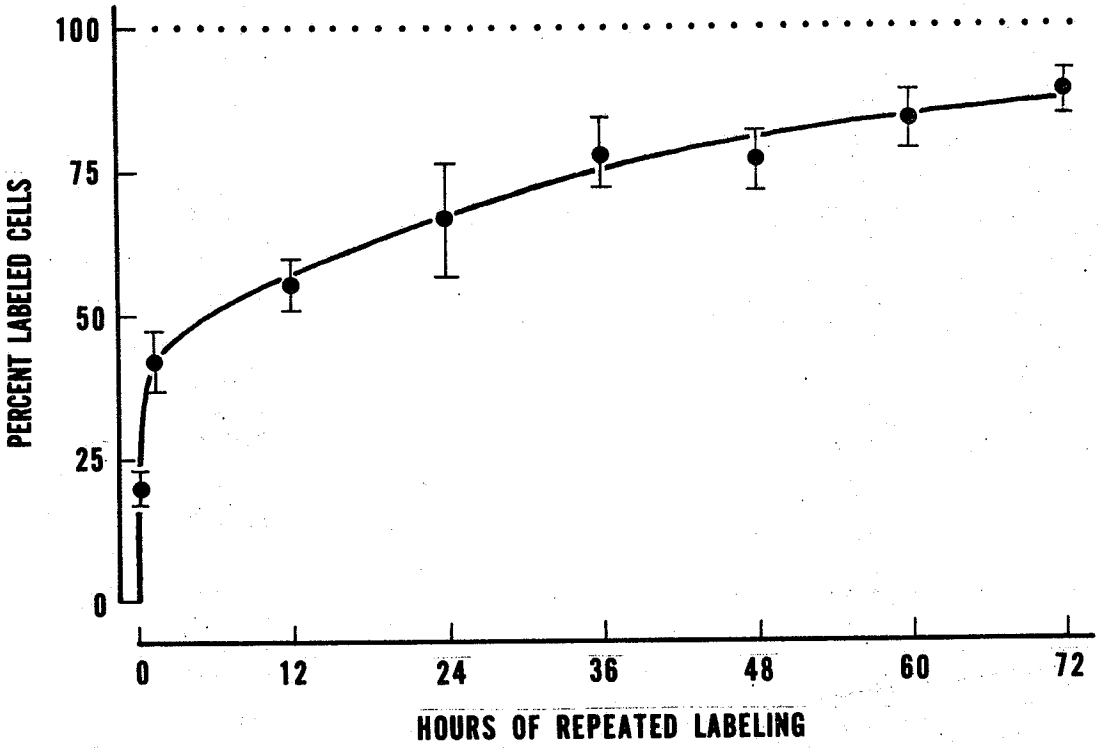

100
75
50
25
0
PERCENT LABELED CELLS
0
12
24
36
48
60
72
HOURS OF REPEATED LABELING

Chart 8

TABLE 9

PERCENTAGE LABELED MITOSES OF DAY 6
B16 MELANOMA[a]

Hours After H^3-Thymidine Injection	Percentage of Labeled Mitoses[b]				Mean ± S.D.
1	16	30	35	21	25.50 ± 7.43
3	87	91	96		91.33 ± 3.76
5	97	94	93	88	93.00 ± 3.24
7	91	97	94		94.00 ± 2.45
9	80	82	95	87	86.00 ± 5.79
11	45	48	50		47.66 ± 2.20
13	46	56	51		51.00 ± 4.08
15	74	71	64	75	71.00 ± 4.30
17	76	88	74	79	79.25 ± 5.36
19	84	74	78	80	79.00 ± 3.61
21	73	75	67	59	68.50 ± 6.22
23	76	72	64	55	66.75 ± 8.04
25	57	64	65	71	64.25 ± 4.97
27	68	80	73		73.66 ± 5.02

[a] data plotted in Chart 9a.

[b] each figure represents a single tumor.

Chart 9.

Labeled mitoses curves of B16 melanoma from animals killed at 2 hour intervals after a pulse label of H^3-thymidine. A. On day 6 of tumor growth, and B. on day 18 of tumor growth. Each point represents the data from a single tumor. The solid lines are drawn through the means of each group.

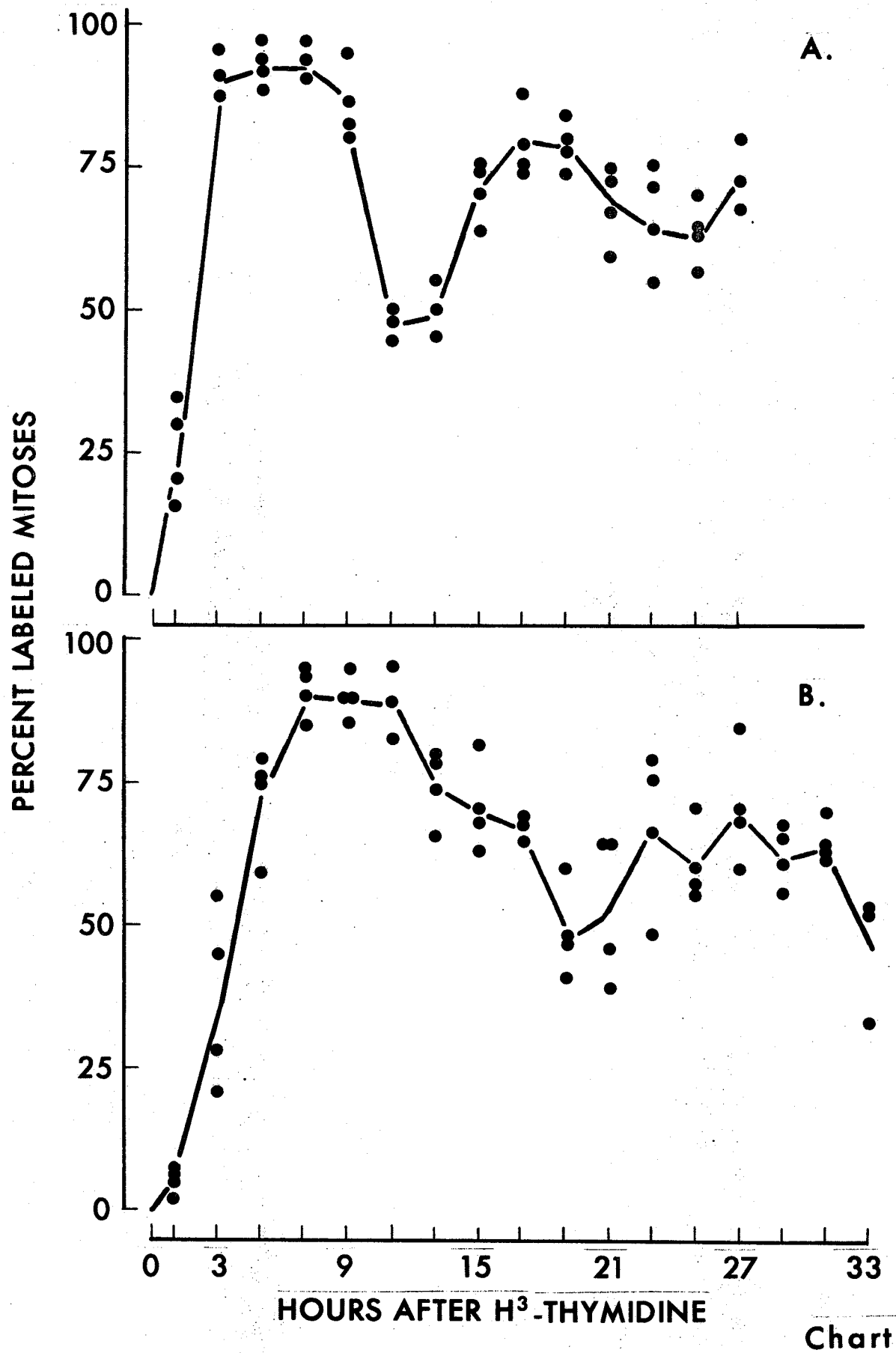

A.
B.
100
75
50
25
0
PERCENT LABELED MITOSES
0
3
9
15
21
27
33
HOURS AFTER H³-THYMIDINE

Chart 9

the duration of mitosis ($\frac{1}{2}M$) could be determined to be 1.5 hours by the following calculation: $G_1 + \frac{1}{2}M = t_C - (S + G_2 + \frac{1}{2}M)$. The duration of G_2 = 1.5 hours - 0.45 hr. ($\frac{1}{2}M$) = 1.05 hrs. and the duration of G_1 = 1.5 hours - 0.45 hr. ($\frac{1}{2}M$) = 1.05 hrs.

To determine the cell cycle time of day 18 tumors, 65 tumor bearing mice received a single i.p. injection of H^3-thymidine at 10 a.m. on day 18 of tumor growth. Groups of 3-4 tumor bearing mice were killed at 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31 and 33 hours after the administration of the tracer. The percentage of labeled mitoses was determined from autoradiography for each tumor. The individual tumor values with their group means are listed in Table 10 and illustrated in Chart 9B. The total cell cycle time (t_C) of B16 melanoma cells on day 18 of tumor growth was estimated from these data to be 19.5 hours. The duration of the S phase was 15.5 hours, that of premitotic gap plus one-half the duration of mitosis was 3.5 hours. t_M was calculated to be 1.54 hours. From these data, the duration of $G_1 + \frac{1}{2}M$ was determined to be 0.5 hours by the following calculation: $G_1 + \frac{1}{2}M = t_C - (S + G_2 + \frac{1}{2}M)$. Therefore, the duration of G_2 = 3.5 hours - 0.75 hr. ($\frac{1}{2}M$) = 2.75 hours and the duration of G_1 = 0.5 hrs. - 0.75 hr ($\frac{1}{2}M$) = 0 hours.

TABLE 10

PERCENTAGE LABELED MITOSES OF DAY 18
B16 MELANOMA[a]

Hours After H^3-Thymidine Injection	Percentage of Labeled Mitoses[b]				Mean ± S.D.
1	2	6	7	8	5.75 ± 2.28
3	45	28	21	56	37.50 ± 13.79
5	74	76	59	79	72.00 ± 7.65
7	86	90	95	94	91.25 ± 3.56
9	86	90	90	96	90.50 ± 3.57
11	89	83	96		89.33 ± 5.37
13	66	74	80	79	74.75 ± 5.54
15	82	68	63	71	71.00 ± 6.96
17	69	68	65		67.33 ± 1.82
19	41	48	60	47	49.00 ± 6.89
21	39	64	64	46	53.25 ± 11.03
23	76	48	67	79	67.50 ± 12.09
25	56	60	57	71	61.00 ± 5.96
27	68	60	71	85	71.00 ± 9.03
29	68	56	66	61	62.75 ± 4.66
31	70	62	64	63	64.75 ± 3.12
33	52	53	33		46.00 ± 9.20

[a]data plotted in Chart 9b.

[b]each figure represents a single tumor.

Section II

This section deals with the production of cell synchrony employing varying dosages, numbers of injections and time intervals between the multiple administrations of ara-C.

As controls for the different synchrony series, 4 groups of 4 tumor bearing mice each, served parallel to each of the first four series. These 16 groups of tumor bearing mice were killed after receiving a 15 minute pulse label, at 2 hour intervals beginning with 8 a.m. of day 9 of tumor growth through to 2 p.m. of day 11. They thus covered in chronological order a total period of 30 hours. The data are listed in Table 11. The LI_{15} ranged from 13.69 to 24.61% without any significant difference between the 16 groups ($p > 0.05$). The mean LI_{15} was 18.60 ± 5.08%. Accordingly, diurnal variantion of DNA synthesis did not exist in the B16 melanoma tumors, as evident from the H^3-thymidine uptake. The MI ranged from 1.64 to 2.66% with a mean value of 2.18 ± 0.50%. The DI ranged from 0.46 to 1.56% with a mean of 0.91 ± 0.52%. Neither the MI nor the DI revealed significant variations, and therefore diurnal variations were not observed.

SERIES 1

Synchrony was attempted in this series by 4 i.p. injections of ara-C at 4 hour intervals with a dosage of 12.5 mg/kg. Ara-C was administered to 11 groups of tumor bearing mice at 10 p.m., 2 a.m., 6 a.m., and 10 a.m. on day 10 of tumor growth. The LI_{15} of each group is listed in Table 12 and graphically portrayed in Chart 10. The LI_{15} of the group killed 4 hours after the last ara-C injection was 6.17%, and thus well below the control level of 18.6%. The LI_{15} of the 6

TABLE 11

PERCENTAGES OF LABELED CELLS, MITOTIC INDEX, AND DEGENERATING INDEX OF UNTREATED B16 MELANOMA BEFORE, DURING, AND FOLLOWING DAY 10 OF TUMOR GROWTH

Time of Day	Number of Animals	Labeling Index (% ± S.D.)	Mitotic Index (% ± S.D.)	Degenerating Index (% ± S.D.)
8 am	4	13.69 ± 4.47	2.10 ± 0.73	0.46 ± 0.10
10 am*	3	20.20 ± 2.02	2.48 ± 0.59	1.09 ± 0.97
12 md*	4	21.62 ± 2.08	2.14 ± 0.55	0.61 ± 0.23
2 pm*	4	13.99 ± 5.59	2.20 ± 0.65	0.93 ± 0.16
4 pm*	4	18.63 ± 5.67	1.97 ± 0.57	1.03 ± 0.71
6 pm*	4	18.28 ± 6.66	2.22 ± 0.44	0.98 ± 0.26
8 pm*	4	15.49 ± 2.56	1.64 ± 0.30	1.02 ± 0.57
10 pm*	4	24.61 ± 5.81	2.05 ± 0.36	0.71 ± 0.99
12 mn*	4	17.20 ± 4.30	2.04 ± 0.28	0.70 ± 0.28
2 am*	3	21.02 ± 1.72	2.10 ± 0.30	0.71 ± 0.46
4 am*	4	21.55 ± 5.94	2.45 ± 0.43	0.89 ± 0.54
6 am*	4	15.60 ± 5.40	2.36 ± 0.58	0.76 ± 0.37
8 am*	4	19.87 ± 4.91	2.21 ± 0.21	0.95 ± 0.18
10 am	4	20.41 ± 5.48	2.66 ± 0.32	1.56 ± 0.71
12 md	4	17.55 ± 4.68	2.37 ± 0.84	0.90 ± 0.56
2 pm	4	18.85 ± 4.37	1.89 ± 0.24	1.16 ± 0.24
Mean % ± S.D.		18.60 ± 5.08	2.18 ± 0.50	0.91 ± 0.52

*= Day 10 of Tumor Growth, md = midday, mn = midnight.

TABLE 12

PERCENTAGES OF LABELED CELLS, MITOTIC INDEX, AND DEGENERATING INDEX OF B16 MELANOMA AT DIFFERENT INTERVALS AFTER FOUR I.P. INJECTIONS OF ARA-C (12.5 mg/kg) ADMINISTERED AT FOUR HOUR INTERVALS

Hours After Last Ara-C Injection	Animals/ Group	Percentage of[a] Labeled Cells (mean ± S.D.)	Mitotic[a] Index (mean ± S.D.)	Degenerating[b] Index (mean ± S.D.)
4	4	6.71 ± 5.86	0.43 ± 0.34	5.16 ± 1.56
6	4	5.95 ± 2.55	0.07 ± 0.04	2.61 ± 0.77
8	4	13.27 ± 10.00	0.12 ± 0.04	4.58 ± 0.63
10	4	7.48 ± 4.84	1.49 ± 1.42	4.06 ± 2.62
12	4	4.72 ± 0.15	2.28 ± 0.56	4.79 ± 1.08
14	4	4.08 ± 1.35	2.58 ± 0.71	5.94 ± 1.34
16	4	8.74 ± 2.71	1.63 ± 0.10	5.37 ± 0.77
18	4	14.18 ± 6.69	1.95 ± 0.47	5.74 ± 3.18
20	4	13.46 ± 2.03	1.58 ± 0.42	2.70 ± 1.81
22	4	17.11 ± 2.41	1.76 ± 0.12	3.44 ± 1.10
24	4	11.51 ± 2.55	1.90 ± 0.48	2.42 ± 0.98

[a] graphically illustrated in Chart 10.

[b] graphically illustrated in Chart 11a.

Chart 10.

Labeling and mitotic index of B16 melanoma at different intervals following 4 i.p. injections of 12.5 mg/kg of ara-C administered at 4 hour intervals. The synchrony index (SI) for this series was 9.2. ⊙ represents control levels and the arrows indicate the points of ara-C injections.

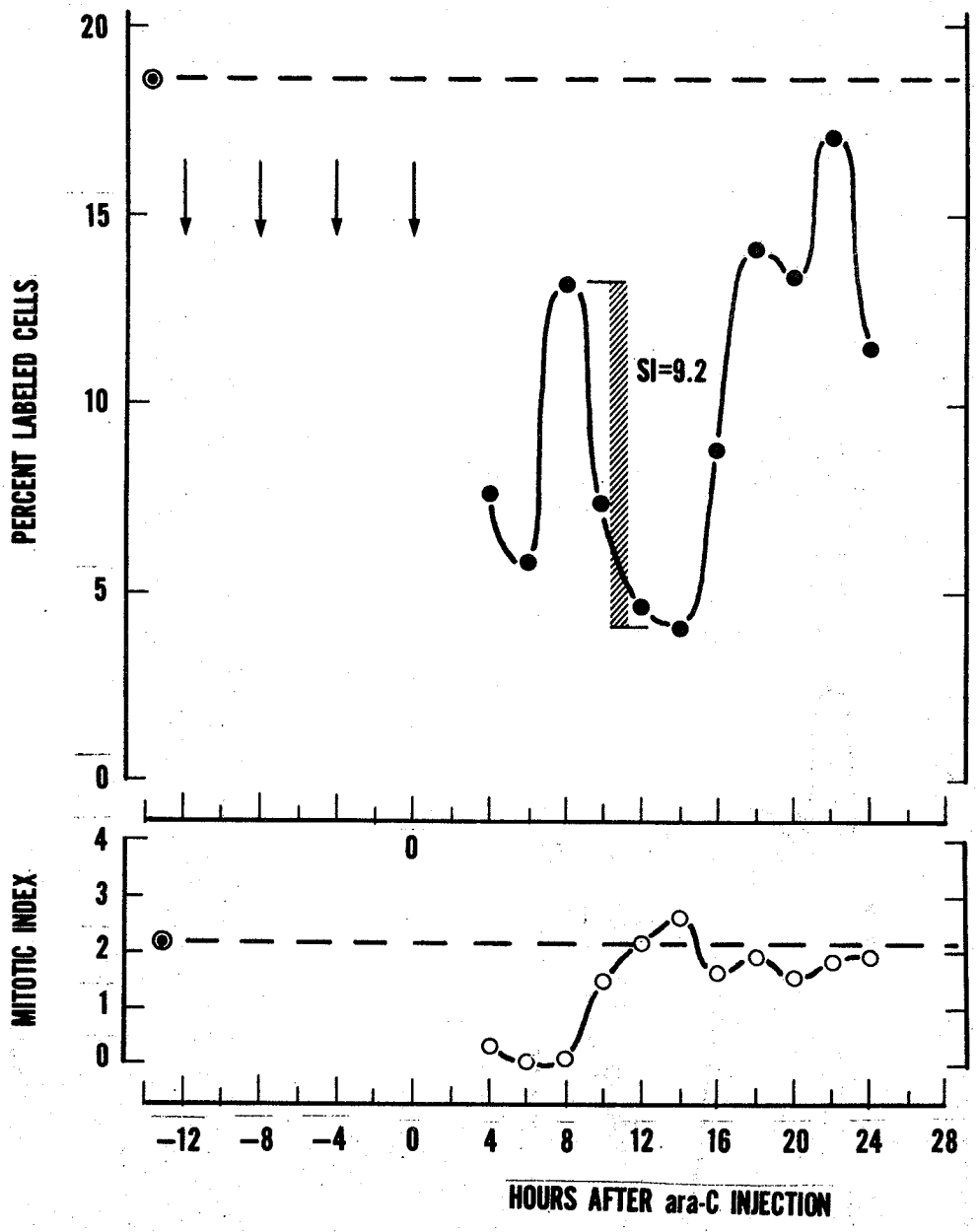

PERCENT LABELED CELLS
20
15
10
5
0
SI=9.2
MITOTIC INDEX
4
3
2
1
0
0
−12
−8
−4
0
4
8
12
16
20
24
28
HOURS AFTER ara-C INJECTION

Chart 10

hour group had declined further to 5.95%, while the LI_{15} of the 8 hour group had risen to 13.27%. From the 8 hour group onward, the LI_{15} of the 10, 12 and 14 hour groups gradually declined again attaining a low of 4.08% in the 14 hour group. The LI_{15} of the 22 hour group augmented to 17.1%, but declined again in the 24 hour group to 11.5% (Chart 10). Although the highest in this series, the LI_{15} of the 22 hour group did not reach the normal control level. The synchrony index was determined in this series to be 9.2.

The MI and DI data of each experimental group following the last injection of ara-C, are listed in Table 12. The MI is further illustrated graphically in Chart 10, the DI in Chart 11A. The MI remained near the zero mark until 10 hours after the last ara-C injection when it rose to 1.49%. In the subsequent groups the MI continued to rise, overshooting the control MI of 2.18%, and attaining the maximum of 2.58%, 14 hours after the last ara-C injection. Following this peak, the MI of the last 5 groups oscillated just below the control level.

The DI remained in all groups well above the mean control DI of 0.91% (Chart 11A). In the first group, 4 hours after the last ara-C injection, the DI was 5.16%, in the next group it declined to 2.61% but increased in the subsequent groups attaining the maximum of 5.94% 14 hours after the last ara-C administration. There was a decline of the DI in the following groups and it never again reached the control level.

Series 2

Synchrony was attempted in this series by the use of 4 i.p. injections of ara-C at 4 hour intervals with a dosage of 25.0 mg/kg.

Chart 11.

Degenerating index vs. time after the last ara-C injection.

(a) 4 injections of ara-C (12.5 mg/kg) at 4 hour intervals;

(b) 4 injections of ara-C (25 mg/kg) at 4 hour intervals;

(c) 8 injections of ara-C (12.5 mg/kg) at 2 hour intervals; and

(d) 8 injections of ara-C (12.5 mg/kg) at 1 hour intervals.

Each point represents the mean of 4 tumors.

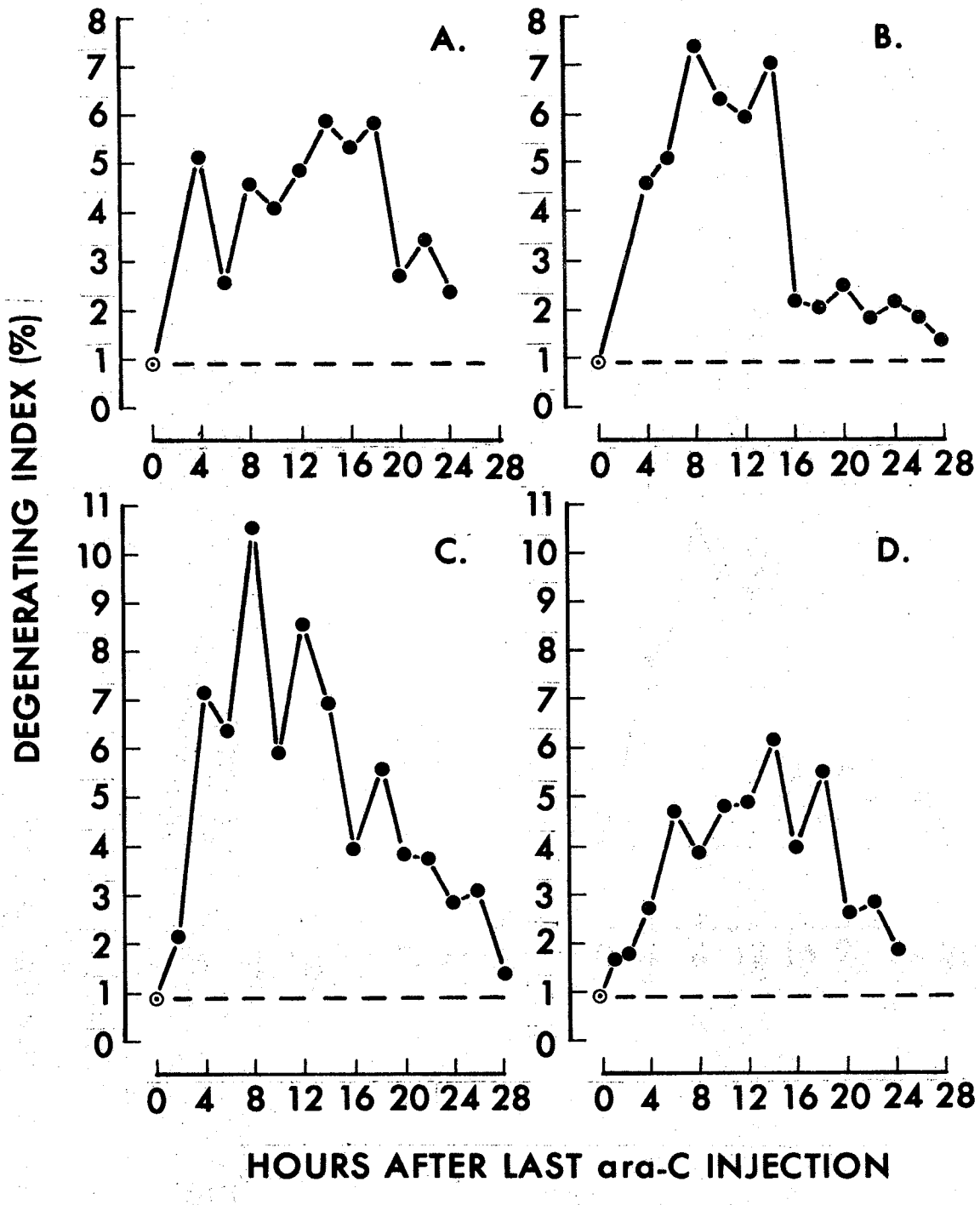

A.
B.
C.
D.
DEGENERATING INDEX (%)
HOURS AFTER LAST ara-C INJECTION
0 4 8 12 16 20 24 28

Chart 11

Ara-C was administered to 13 groups of tumor bearing mice at 10 p.m., 2 a.m., 6 a.m., and 10 a.m. on day 10 of tumor growth. The LI_{15} data of each group are listed in Table 13 and illustrated in Chart 12. The LI_{15} of the group killed 4 hours after the last ara-C injection was 8.86%, well below the control level of 18.6%. The LI_{15} of the following group declined still further to 6.66%. Subsequently, the LI_{15} of the group killed 8 hours after the last injection of ara-C rose to 15.04% and four hours later the LI_{15} declined again to 5.8%. Six hours later the LI_{15} rose to 12.87%, 18 hours after the last injection of ara-C. The LI_{15} of the following groups oscillated, reaching a peak of 17.8% approaching the control level of 18.6%; in the second last group, the LI_{15} again declined to 12.85% (Chart 12). The synchrony index was determined to be 9.2.

The MI and DI data of each of the experimental groups, in this series, are listed in Table 13. The MI data of the tumor groups at 4, 6, 8 and 10 hours after the last injection of ara-C remained near the zero level (Chart 12). Twelve hours after the last injection of ara-C, the MI rose to 0.99% and continued to augment in the subsequent groups until reaching a maximum in the 20 hour group of 2.95%. The MI then declined to 1.43%, that is, 2 hours after the peak level. The MI of the later groups oscillated about the 2.18% control level.

The DI data are graphically presented in Chart 11B. The DI of the first group, 4 hours after the last ara-C injection, was 4.62%, well above the control DI (0.91%). Four hours later the DI was 7.45% and remained near this value for the next six hours. Sixteen hours after the last ara-C injection, the DI fell to 2.34%. The DI of the following groups gradually declined further, approaching the control

TABLE 13

PERCENTAGES OF LABELED CELLS, MITOTIC INDEX, AND DEGENERATING INDEX OF B16 MELANOMA AT DIFFERENT INTERVALS AFTER FOUR I.P. INJECTIONS OF ARA-C (25 mg/kg) ADMINISTERED AT FOUR HOUR INTERVALS

Hours After Last Ara-C Injection	Animals/ Group	Percentage of[a] Labeled cells (mean ± S.D.)	Mitotic[a] Index (mean ± S.D.)	Degenerating[b] Index (mean ± S.D.)
4	4	8.86 ± 1.09	0.04 ± 0.05	4.62 ± 2.51
6	4	6.66 ± 2.31	0.16 ± 0.12	5.07 ± 1.60
8	4	15.04 ± 2.72	0.40 ± 0.05	7.45 ± 3.42
10	4	14.40 ± 2.95	0.19 ± 0.08	6.30 ± 1.97
12	4	5.80 ± 1.30	0.99 ± 0.74	5.89 ± 2.10
14	4	8.90 ± 3.22	1.50 ± 1.31	7.05 ± 4.98
16	4	11.03 ± 3.62	1.41 ± 0.76	2.34 ± 1.02
18	4	12.87 ± 2.30	2.42 ± 0.80	2.02 ± 0.49
20	4	9.58 ± 5.26	2.95 ± 0.95	2.55 ± 1.10
22	4	10.12 ± 3.17	1.43 ± 0.24	1.73 ± 0.90
24	3	17.83 ± 9.20	2.48 ± 1.10	2.27 ± 1.48
26	4	12.85 ± 7.77	2.07 ± 0.57	1.74 ± 1.12
28	4	14.51 ± 3.03	1.96 ± 0.29	1.46 ± 0.23

[a] graphically illustrated in Chart 12.

[b] graphically illustrated in Chart 11b.

Chart 12.

Labeling and mitotic index of B16 melanoma at different intervals following 4 i.p. injections of a 25 mg/kg dose of ara-C administered at 4 hour intervals. The synchrony index (SI) of this series was 9.2. ⊙ represents control levels and the arrows indicate the points of ara-C injections.

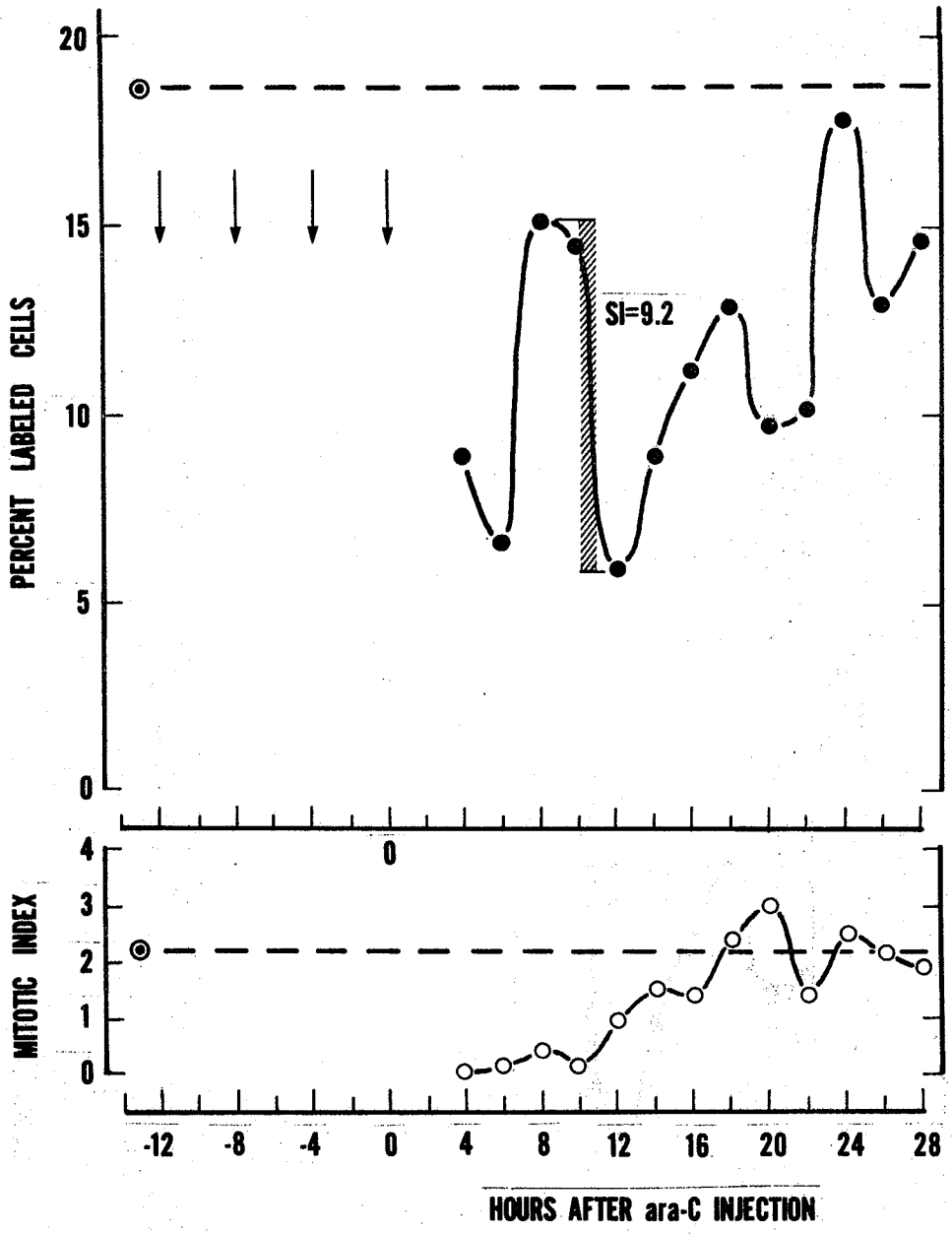

PERCENT LABELED CELLS
20
15
10
5
0
SI=9.2
MITOTIC INDEX
4
3
2
1
0
-12
-8
-4
0
4
8
12
16
20
24
28
HOURS AFTER ara-C INJECTION

Chart 12

level but never reached it (0.91%).

SERIES 3

In this series synchrony was attempted by the injection of 8 single i.p. injections of ara-C at 1 hour intervals of a dosage of 12.5 mg/kg. Ara-C was administered to 13 groups of tumor bearing mice at 10 p.m., 11 p.m., 12 midnight, 1 a.m., 2 a.m., 3 a.m., 4 a.m. and 5 a.m. on the 10th day of tumor growth. The LI_{15} data of each group are presented in Table 14 and plotted in Chart 13. The LI_{15} of the first two groups killed 1 and 2 hours after the last injection of ara-C exhibited the lowest level, 0.24 and 0.46%. Beginning with the group killed 4 hours after the last injection of ara-C, the LI_{15} increased steadily over the next two groups, attaining a maximum level after 8 hours of 23.62%. Subsequently, the LI_{15} of the following groups declined to an eventual low of 6.25%, 18 hours after the last ara-C injection. Statistically, the mean LI_{15} of the 14 and 16 hour groups were the same ($p > 0.05$). Following the 18 hour group, the LI_{15} of the three remaining groups increased and reached the 16.64% level by 24 hours. The synchrony index was 17.3. The 18 hour group served as the L_{min} because the 14 and 16 hour groups were statistically similar.

The MI and DI data of the experimental groups in this series are listed in Table 14. The MI of the first 5 groups remained below the 0.36% level (Chart 13). It increased steadily in the next 4 groups reaching 3.31% 16 hours after the last injection of ara-C. The MI of the 18 hour group declined to the control level. The MI values of the following groups once again peaked at 3.73%. In the

TABLE 14

PERCENTAGES OF LABELED CELLS, MITOTIC INDEX AND DEGENERATING INDEX OF B16 MELANOMA AT DIFFERENT INTERVALS AFTER EIGHT I.P. INJECTIONS OF ARA-C (12.5 mg/kg) ADMINISTERED AT ONE HOUR INTERVALS

Hours After Last Ara-C Injection	Animals/ Group	Percentage of[a] Labeled Cells (mean ± S.D.)	Mitotic[a] Index (mean ± S.D.)	Degenerating[b] Index (mean ± S.D.)
1	4	0.24 ± 0.23	0.02 ± 0.04	1.62 ± 0.65
2	4	0.46 ± 0.26	0.00	1.68 ± 0.91
4	4	3.19 ± 3.08	0.11 ± 0.14	2.66 ± 1.31
6	4	14.03 ± 5.37	0.06 ± 0.08	4.69 ± 2.42
8	4	23.62 ± 0.11	0.35 ± 0.30	3.84 ± 1.50
10	4	20.24 ± 4.45	0.90 ± 0.70	4.77 ± 1.73
12	4	10.68 ± 6.67	0.82 ± 0.85	4.92 ± 0.82
14	4	8.94 ± 3.20	1.74 ± 2.03	6.19 ± 2.15
16	4	10.45 ± 6.13	3.31 ± 1.61	3.92 ± 1.96
18	4	6.25 ± 3.66	1.92 ± 0.84	5.54 ± 1.65
20	4	11.20 ± 5.33	3.73 ± 0.32	2.50 ± 1.16
22	4	14.67 ± 6.83	2.61 ± 0.53	2.92 ± 1.38
24	4	16.64 ± 3.13	2.46 ± 0.57	1.74 ± 0.40

[a] graphically illustrated in Chart 13.

[b] graphically illustrated in Chart 11d.

Chart 13.

Labeling and mitotic index of B16 melanoma at different intervals following 8 i.p. injections of a 12.5 mg/kg dose of ara-C administered at 1 hour intervals. The synchrony index (SI) of this series was 17.3. ⊙ represents control levels and the arrows indicate the periods of ara-C injections.

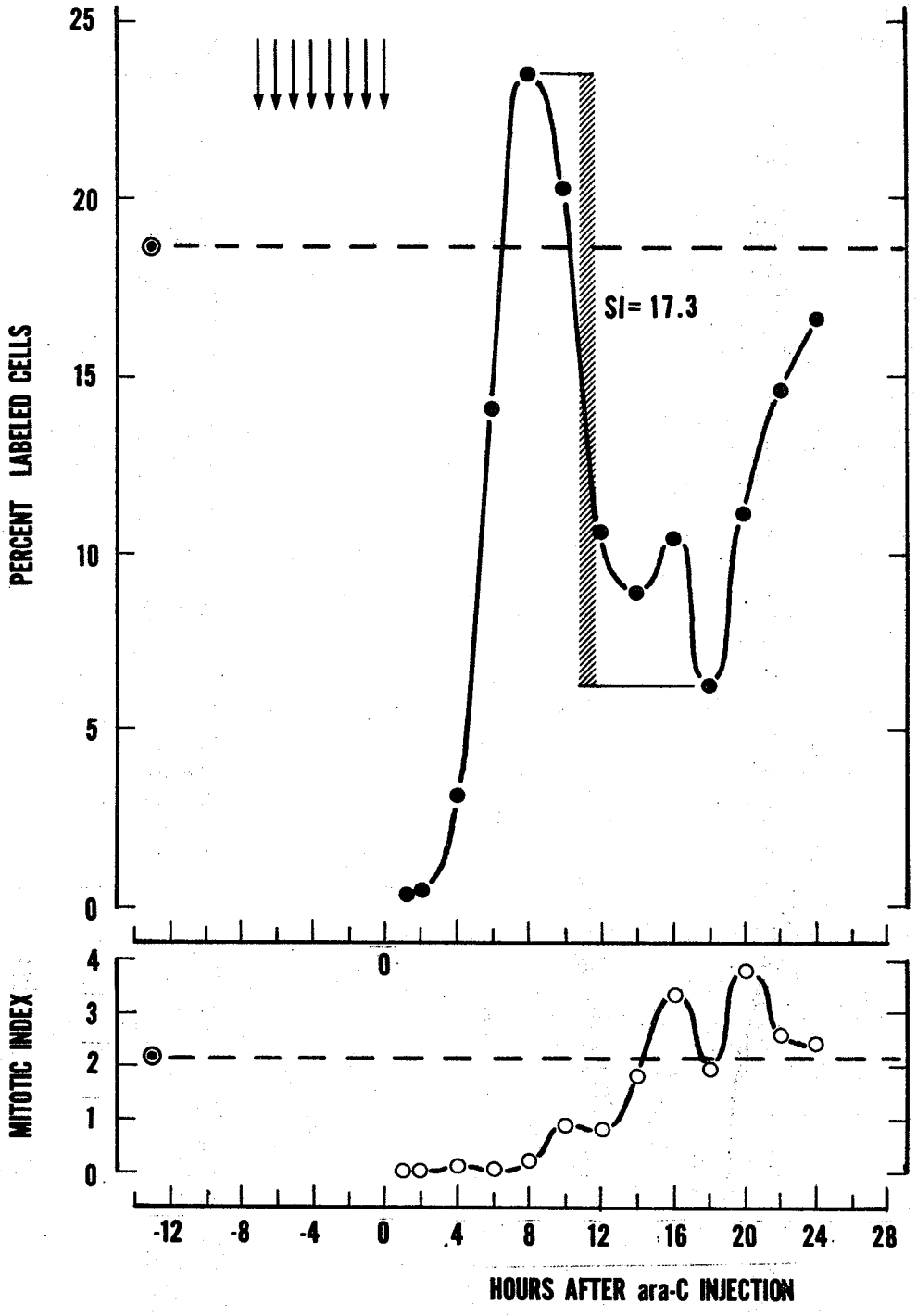

25
20
15
10
5
0
PERCENT LABELED CELLS
SI= 17.3
0
4
3
2
1
0
MITOTIC INDEX
-12
-8
-4
0
4
8
12
16
20
24
28
HOURS AFTER ara-C INJECTION

Chart 13

remaining two groups the MI declined to 2.61 and 2.46% respectively.

The DI data of the different groups are presented in Chart 11D. In all instances the DI was above the control DI of 0.91%. The DI of the groups became steadily augmented until attaining a maximum of 6.19% in the 14 hour group. The DI values of the later groups, oscilated, declined eventually and approached the control level by 24 hours; that is 1.74%.

SERIES 4

Synchrony was attempted in this series by the use of 8 i.p. injections of ara-C at 2 hour intervals with a dosage 12.5 mg/kg. Ara-C was administered to 14 groups of tumor bearing mice at 10 p.m., 12 midnight, 2 a.m., 4 a.m., 6 a.m., 8 a.m., 10 a.m., 12 noon on the 10th day of tumor growth. The mean LI_{15} data of the groups are listed in Table 15 and plotted in Chart 14. The LI_{15} of the 2 hour group was close to the zero level, 2.63%. The LI_{15} of the next four groups, killed 4, 6, 8, and 10 hours after ara-C, augmented gradually to a peak of 45.06%. The mean LI_{15} declined subsequently to a low of 4.4%, 18 hours after the last injection of ara-C. The LI_{15} of the later groups attained the control LI_{15} level (18.6%), and then oscillated about this level (Chart 14). The synchrony index in this series was 40.7.

The MI and DI data for each of the groups of this series are listed in Table 15. The mean MI (Chart 14) for the first 6 groups was lower than the 0.62% level. It increased slowly in the following groups, attaining the peak of 3.49%, 18 hours after the last injection. The mean MI for the remaining 5 groups declined to the control level (2.18%) and oscillated about this level.

TABLE 15

PERCENTAGE OF LABELED CELLS, MITOTIC INDEX AND DEGENERATING INDEX OF B16 MELANOMA AT DIFFERENT INTERVALS AFTER EIGHT I.P. INJECTIONS OF ARA-C (12.5 mg/kg) ADMINISTERED AT TWO HOUR INTERVALS

Hours After Last Ara-C Injection	Animals/ Group	Percentage of[a] Labeled Cells (mean ± S.D.)	Mitotic[a] Index (mean ± S.D.)	Degenerating[b] Index (mean ± S.D.)
2	4	2.63 ± 0.92	0.02 ± 0.04	2.26 ± 1.43
4	4	6.78 ± 4.62	0.08 ± 0.10	7.20 ± 4.90
6	4	20.27 ± 11.82	0.07 ± 0.09	6.44 ± 3.01
8	4	23.16 ± 15.45	0.09 ± 0.13	10.64 ± 6.30
10	4	45.06 ± 3.40	0.61 ± 0.73	5.96 ± 2.15
12	4	39.02 ± 12.58	0.61 ± 0.71	8.57 ± 2.22
14	4	28.74 ± 7.78	1.40 ± 1.23	6.98 ± 1.76
16	4	13.23 ± 9.96	2.83 ± 1.36	3.92 ± 1.64
18	4	4.40 ± 0.75	3.49 ± 1.34	5.61 ± 1.95
20	4	7.25 ± 2.97	2.37 ± 0.71	3.89 ± 1.01
22	4	19.07 ± 3.47	2.22 ± 0.44	3.79 ± 2.11
24	4	20.60 ± 3.21	2.41 ± 0.77	2.76 ± 0.43
26	4	13.20 ± 7.16	2.21 ± 1.17	3.02 ± 2.06
28	4	17.68 ± 3.49	2.48 ± 0.62	1.37 ± 0.57

[a] graphically illustrated in Chart 14.

[b] graphically illustrated in Chart 11c.

Chart 14.

Labeling and mitotic index of B16 melanoma at different intervals following 8 i.p. injections of a 12.5 mg/kg dose of ara-C administered at 2 hour intervals. The synchrony index (SI) of this series was 40.7. ⊙ represents control levels and the arrows indicate the points of ara-C injections.

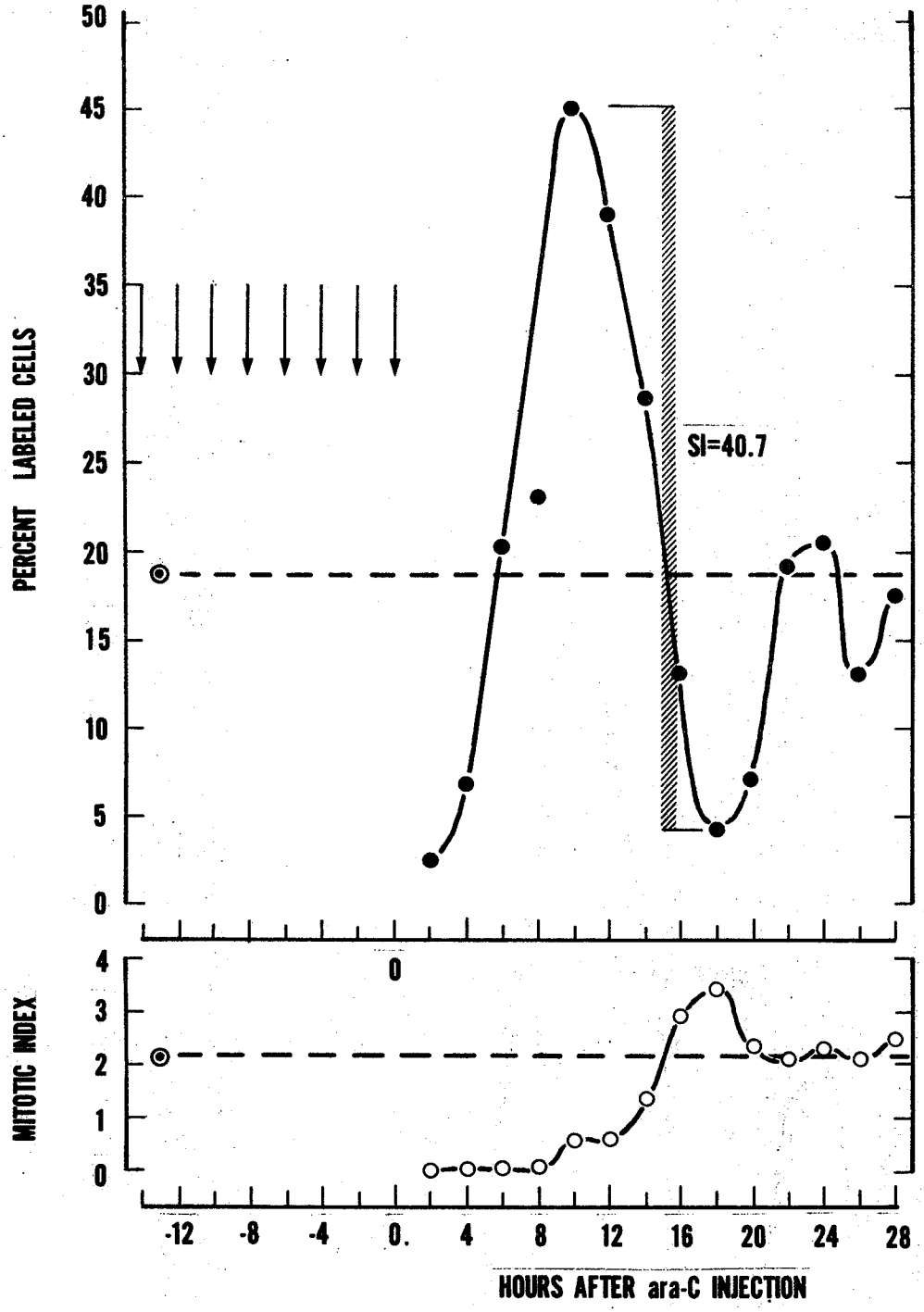

PERCENT LABELED CELLS
50
45
40
35
30
25
20
15
10
5
0
SI=40.7
MITOTIC INDEX
4
3
2
1
0
0
-12
-8
-4
0.
4
8
12
16
20
24
28
HOURS AFTER ara-C INJECTION

Chart 14

The mean DI data of this series are presented in Chart 11C. The DI attained a maximum 10.64%, 8 hours after the last injection of ara-C. Following this peak level, the mean DI of the subsequent groups declined to 1.37%, 28 hours after the last ara-C injection.

SERIES 5

In this series synchrony was attempted by the injection of 8 single i.p. injections of ara-C at 2 hour intervals with a dosage of 18.8 mg/kg. Ara-C was administered into 11 groups of tumor bearing mice at 12 midnight, 2 a.m., 4 a.m., 6 a.m., 8 a.m., 12 noon, and 2 p.m. on day 10 of tumor growth. The LI_{15} data of the groups are listed in Table 16 and plotted in Chart 15. The LI_{15} of the group killed 2 hours after the last ara-C injection was well below the control level, 2.89%, while the LI_{15} of the second group, merely 2 hours later, had risen to 32.27%. The LI_{15} of the 3rd and 4th groups had declined to 10.88% and 9.06%, respectively. The LI_{15} data of the seven remaining groups formed a curve that oscillated about the mean control LI_{15} of 18.6% (Chart 15). The synchrony index was determined in this series to be 23.2.

The MI and DI data of this experimental series are listed in Table 16. The MI (Chart 15) of the first 5 groups remained below 0.23%. The subsequent group, that is, 12 hours after the last injection of ara-C, exhibited a MI of 1.62%, and the MI of the next two groups continued to rise to attain 2.60% and 3.44%, respectively. The MI of the last 3 groups declined gradually to a low of 1.37%.

The DI data of these groups are presented in Chart 16A. All of them were above the control DI level of 0.91%. The maximum DI attained

TABLE 16

PERCENTAGES OF LABELED CELLS, MITOTIC INDEX AND DEGENERATING INDEX OF B16 MELANOMA AT DIFFERENT INTERVALS AFTER EIGHT I.P. INJECTIONS OF ARA-C (18.8 mg/kg) ADMINISTERED AT TWO HOUR INTERVALS

Hours After Last Ara-C Injection	Animals/ Group	Percentage of[a] Labeled Cells (mean ± S.D.)	Mitotic[a] Index (mean ± S.D.)	Degenerating[b] Index (mean ± S.D.)
2	4	2.89 ± 0.22	0.05 ± 0.10	4.14 ± 1.52
4	4	32.27 ± 8.20	0.05 ± 0.10	5.75 ± 2.71
6	4	10.88 ± 5.85	0.12 ± 0.10	4.76 ± 1.74
8	4	9.06 ± 5.46	0.00 ± 0.00	10.16 ± 3.89
10	4	13.43 ± 5.58	0.22 ± 0.26	5.34 ± 2.49
12	4	25.31 ± 9.14	1.62 ± 0.68	4.73 ± 1.41
14	4	17.73 ± 7.62	2.60 ± 3.41	2.99 ± 1.12
16	4	11.20 ± 6.05	3.44 ± 1.97	2.78 ± 1.10
18	4	10.89 ± 3.00	1.76 ± 0.67	3.85 ± 0.73
20	4	20.82 ± 4.99	1.43 ± 0.44	2.23 ± 1.16
22	4	17.94 ± 9.16	1.37 ± 0.65	1.77 ± 0.24

[a] graphically illustrated in Chart 15.

[b] graphically illustrated in Chart 16a.

Chart 15.

Labeling and mitotic index of B16 melanoma at different intervals following 8 i.p. injections of a 18.8 mg/kg dose of ara-C administered at 2 hour intervals. The synchrony index (SI) of this series was 23.2. ⊙ represents control levels and the arrows indicate the periods of ara-C injections.

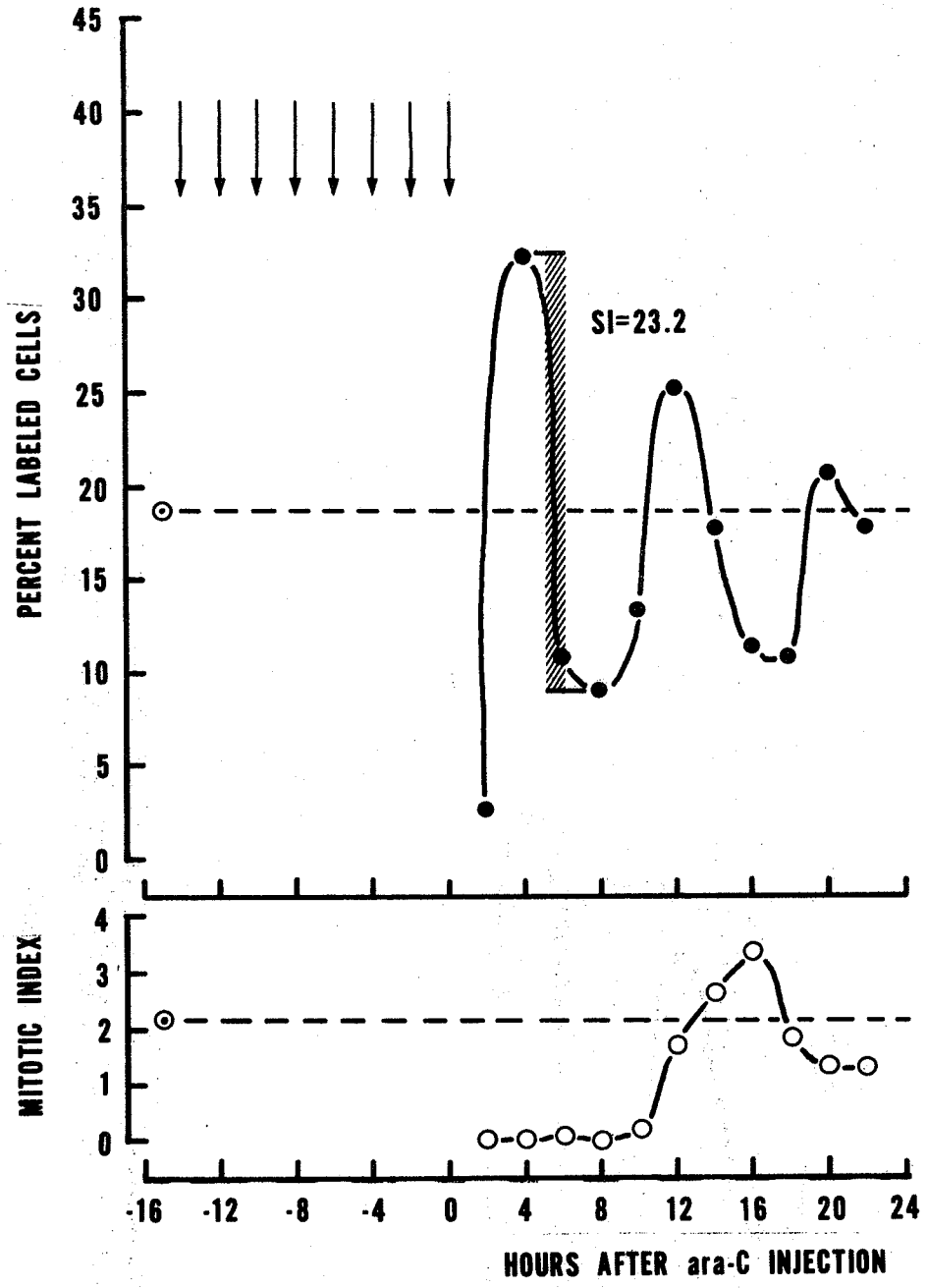

PERCENT LABELED CELLS
45
40
35
30
25
20
15
10
5
0
SI=23.2
MITOTIC INDEX
4
3
2
1
0
-16
-12
-8
-4
0
4
8
12
16
20
24
HOURS AFTER ara-C INJECTION

Chart 15

Chart 16.

Degenerating index vs. time after the last ara-C injection. (a) 8 injections of ara-C (18.8 mg/kg) at 2 hour intervals; (b) 16 injections of ara-C (12.5 mg/kg) at 1 hour intervals; and (c) 16 injections of ara-C (18.8 mg/kg) at 1 hour intervals.

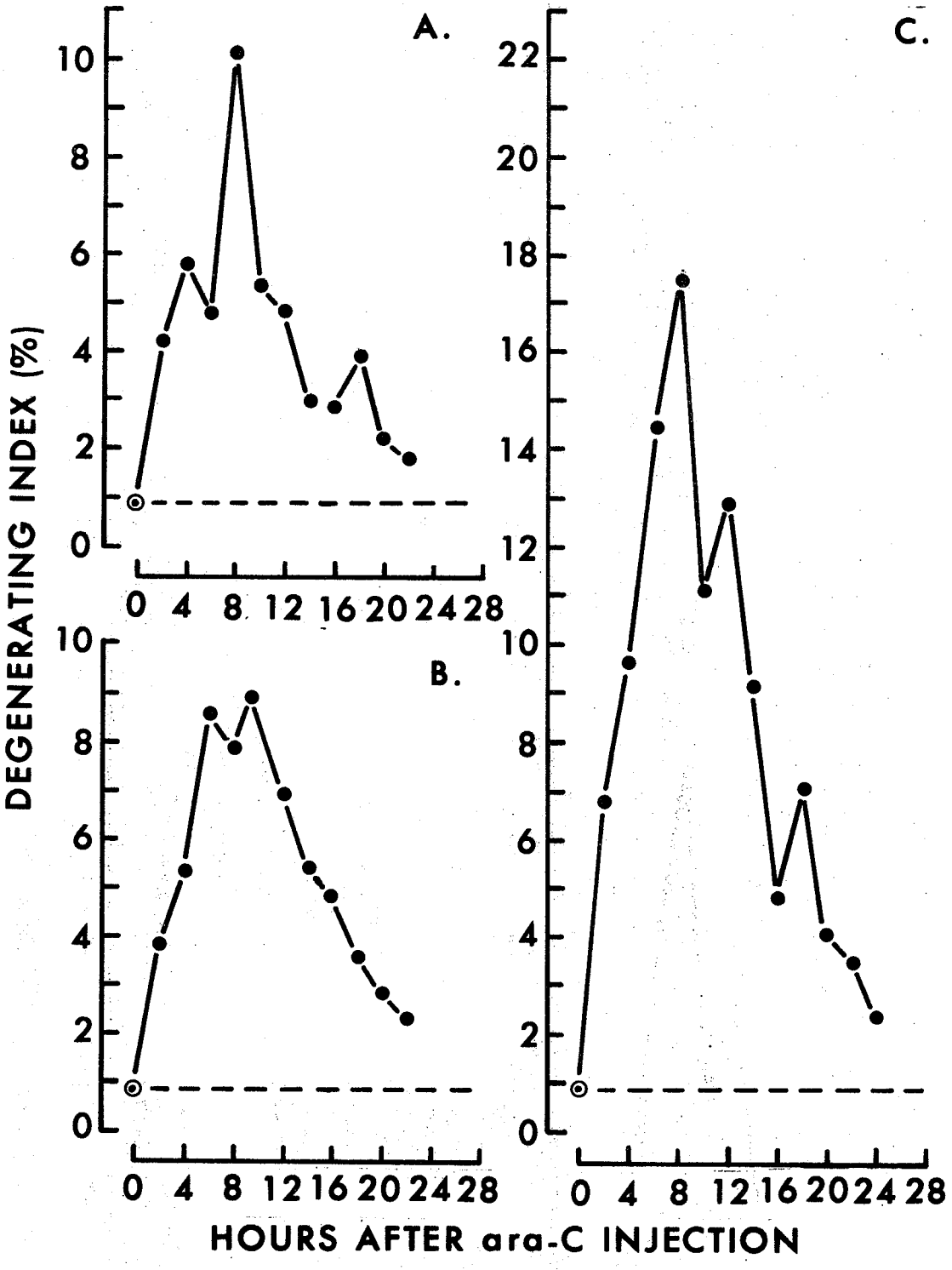

A.
B.
C.
DEGENERATING INDEX (%)
HOURS AFTER ara-C INJECTION
0 4 8 12 16 20 24 28
0 2 4 6 8 10
0 2 4 6 8 10 12 14 16 18 20 22

Chart 16

was 10.16%, 8 hours after the last injection of ara-C. The DI data of the later groups declined gradually, with small oscillations to a low of 1.77%.

SERIES 6

In this series cell synchrony was attempted by 16 i.p. injections of ara-C at 1 hour intervals with a dosage of 12.5 mg/kg. Ara-C was administered to 11 groups of tumor bearing mice on the 10th day of tumor growth, beginning at 9 a.m. and continued every hour for the next 15 hours, with the last injection at 12 noon. The mean LI_{15} data of each group are listed in Table 17 and illustrated in Chart 17. The LI_{15} of the group killed 2 hours after the last injection of ara-C was 6.03%, and the LI_{15} of the 2nd and 3rd groups were 20.21% and 38.61%, respectively. Following the peak LI_{15}, in the third group, the LI_{15} values of the subsequent groups fell to a low of 7.23%, 16 hours after the last ara-C injection. Statistically, the mean LI_{15} of the 12 and 14 hour groups were similar ($p > 0.05$). Subsequent to the 16 hour group, the LI_{15} of the three remaining groups increased, reaching a level of 16.47%, 22 hours after the last injection of ara-C. The synchrony index was determined to be 31.4. The L_{min} of the 16 hour group was employed, as the 12 and 14 hour groups were statistically identical.

The MI and DI data of each of the experimental groups in this series are listed in Table 17. The MI (Chart 17) of the first 5 groups after the last ara-C injection, remained below the 0.43% level. It increased steadily in the next 2 groups, reaching 1.87% 14 hours after the last ara-C injection. The MI of the remaining 4 groups

TABLE 17

PERCENTAGE OF LABELED CELLS, MITOTIC INDEX AND DEGENERATING INDEX OF B16 MELANOMA AT DIFFERENT INTERVALS AFTER 16 I.P. INJECTIONS OF ARA-C (12.5 mg/kg) ADMINISTERED AT ONE HOUR INTERVALS

Hours After Last Ara-C Injection	Animals/ Group	Percentage of[a] Labeled Cells (mean ± S.D.)	Mitotic[a] Index (mean ± S.D.)	Degenerating[b] Index (mean ± S.D.)
2	4	6.03 ± 0.51	0.05 ± 0.10	3.77 ± 1.77
4	4	20.21 ± 13.27	0.10 ± 0.00	5.33 ± 3.04
6	4	38.61 ± 5.69	0.10 ± 0.10	8.59 ± 1.80
8	4	25.80 ± 4.76	0.42 ± 0.24	7.76 ± 3.52
10	4	20.10 ± 8.10	0.40 ± 0.17	8.82 ± 3.90
12	4	11.31 ± 10.16	1.33 ± 1.10	6.91 ± 0.60
14	4	13.21 ± 7.64	1.87 ± 0.89	5.44 ± 0.81
16	3	7.23 ± 2.14	1.10 ± 0.68	4.76 ± 2.36
18	4	9.72 ± 3.75	2.07 ± 1.02	3.59 ± 0.82
20	3	8.62 ± 2.37	1.47 ± 0.52	2.75 ± 0.83
22	4	16.47 ± 8.54	1.84 ± 0.53	2.31 ± 0.51

[a] graphically illustrated in Chart 17.

[b] graphically illustrated in Chart 16b.

Chart 17.

Labeling and mitotic index of B16 melanoma at different intervals following 16 i.p. injections of a 12.5 mg/kg dose of ara-C administered at 1 hour intervals. The synchrony index (SI) for this series was 31.4. ⊙ represents control levels and the arrows indicate the periods of ara-C injections.

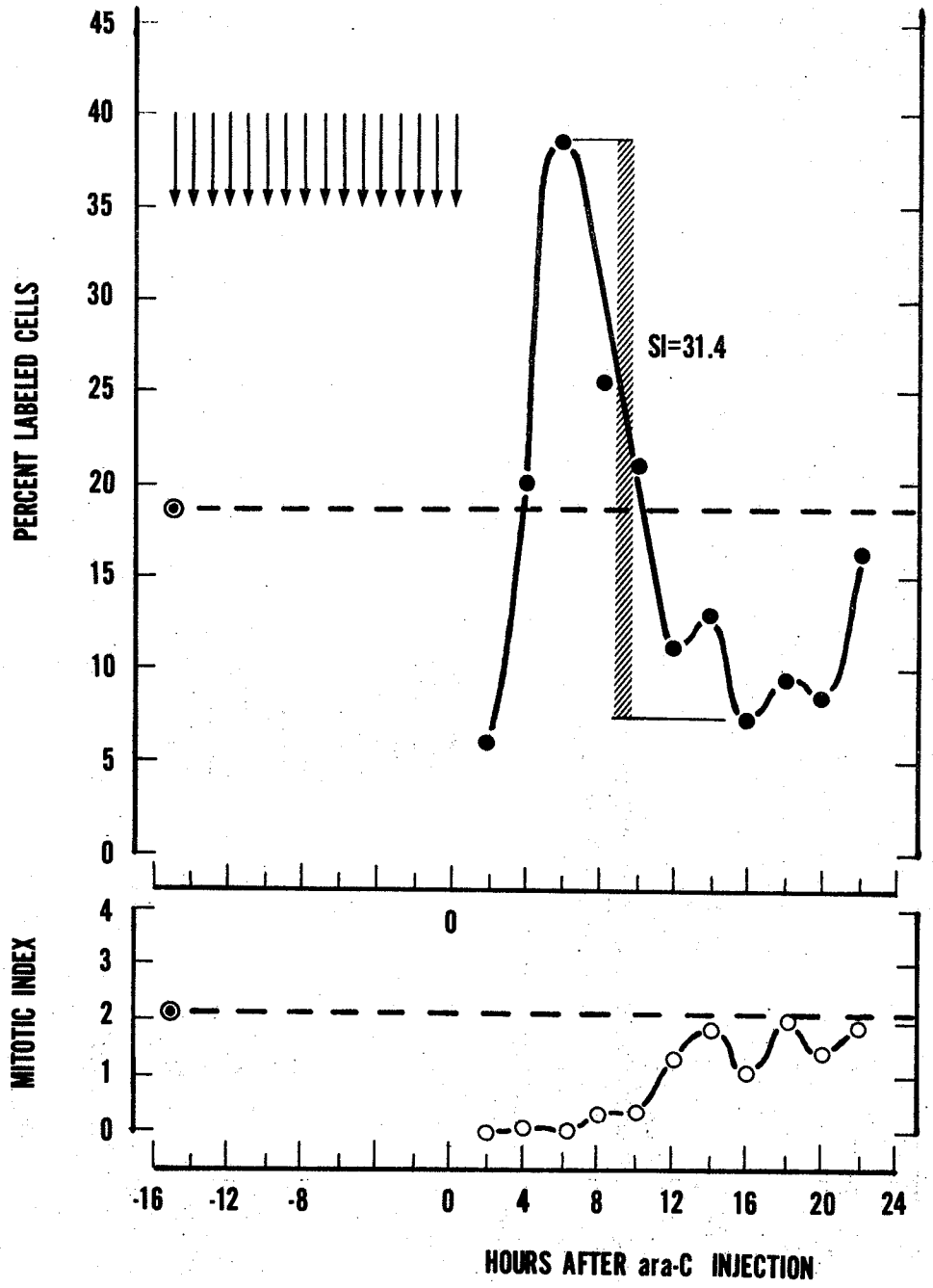

PERCENT LABELED CELLS
45
40
35
30
25
20
15
10
5
0
SI=31.4
MITOTIC INDEX
4
3
2
1
0
0
-16
-12
-8
0
4
8
12
16
20
24
HOURS AFTER ara-C INJECTION

Chart 17

oscillated below the control level (Chart 17). None of the MI values of the treated groups attained the control level of 2.18%.

The DI of the groups in this experimental series are presented in Chart 16B. The DI of all groups was above the control DI level of 0.91%. The DI attained the maximum level of 8.82% 10 hours after the last ara-C injection. In the following 6 groups it declined steadily from this peak level to a low of 2.31%. Within the time limits of this experiment the DI did not return to the control level.

SERIES 7

Synchrony was attempted in this series by 16 i.p. injections of ara-C at 1 hour intervals with a dosage of 18.8 mg/kg. Ara-C was administered to 12 groups of tumor bearing mice at the identical time intervals as in the previous series. The mean LI_{15} data of the groups are listed in Table 18 and plotted in Chart 18. The LI_{15} of the group killed 2 hours after the last ara-C injection was 3.26%. The LI_{15} of the next group came closer to the control level; the LI_{15} peak was reached by the third group, 38.9%. Subsequently, the LI_{15} curve declined to a low of 5.23%, 12 hours after the last injection of ara-C. The LI_{15} of next groups increased steadily to 15.93%, 18 hours after the last ara-C injection. The LI_{15} of the last 3 groups fluctuated around the 14.5% level, just below the control level of 18.6% (Chart 18). The synchrony index was determined to be 33.7.

The MI and DI data of this series are listed in Table 18. The mean MI (Chart 18) of the first group was 0.05%. From the next group onward, the MI increased steadily to a peak of 3.40%, 14 hours after the last ara-C injection. The MI values of the subsequent groups

TABLE 18

PERCENTAGE OF LABELED CELLS, MITOTIC INDEX AND DEGENERATING INDEX OF B16 MELANOMA AT DIFFERENT INTERVALS AFTER 16 I.P. INJECTIONS OF ARA-C (18.8 mg/kg) ADMINISTERED AT 1 HOUR INTERVALS

Hours After Last Ara-C Injection	Animals/ Group	Percentage of[a] Labeled Cells (mean ± S.D.)	Mitotic[a] Index (mean ± S.D.)	Degenerating[b] Index (mean ± S.D.)
2	4	3.26 ± 0.60	0.05 ± 0.10	6.72 ± 1.33
4	4	17.08 ± 10.86	0.07 ± 0.10	9.63 ± 3.23
6	4	38.90 ± 5.94	0.24 ± 0.28	14.48 ± 6.82
8	4	16.44 ± 8.88	0.26 ± 0.10	17.47 ± 3.25
10	4	5.23 ± 3.90	0.85 ± 0.46	11.18 ± 1.94
12	4	5.56 ± 4.00	1.47 ± 1.34	12.90 ± 2.44
14	4	8.23 ± 4.77	3.40 ± 1.05	9.12 ± 3.61
16	4	12.95 ± 1.87	2.27 ± 0.20	4.76 ± 2.10
18	4	15.93 ± 4.80	1.99 ± 1.11	7.07 ± 1.77
20	4	14.58 ± 3.69	1.60 ± 0.56	4.09 ± 0.93
22	4	14.44 ± 4.72	1.93 ± 0.84	3.49 ± 0.78
24	4	15.65 ± 3.25	2.15 ± 0.40	2.37 ± 0.56

[a] graphically illustrated in Chart 18.

[b] graphically illustrated in Chart 16c.

Chart 18.

Labeling and mitotic index of B16 melanoma at different intervals following 16 i.p. injections of a 18.8 mg/kg dose of ara-C administered at 1 hour intervals. The synchrony index (SI) of this series was 33.7. ⊙ represents control levels and the arrows indicate the periods of ara-C injections.

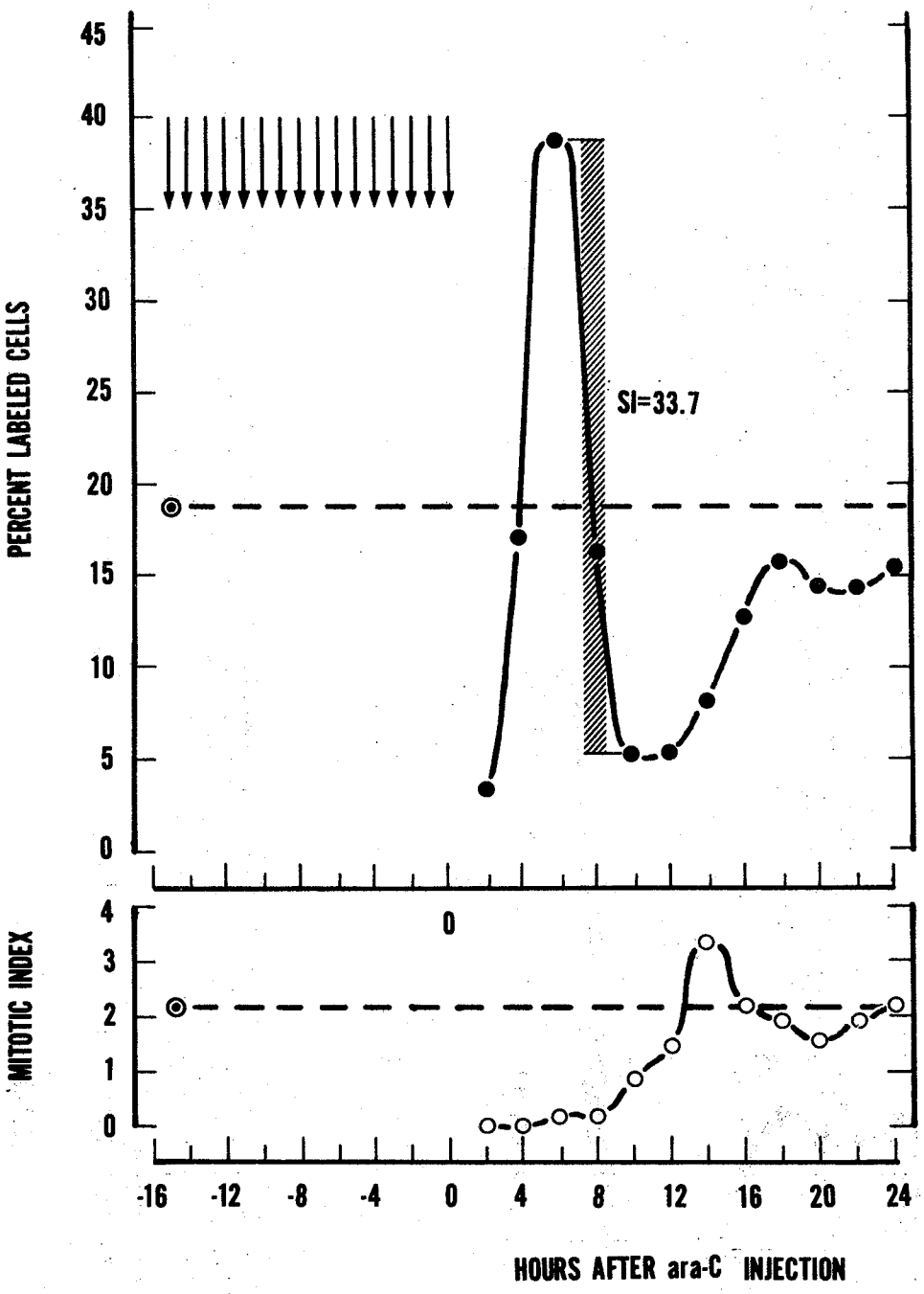

PERCENT LABELED CELLS
45
40
35
30
25
20
15
10
5
0
SI=33.7
MITOTIC INDEX
4
3
2
1
0
0
-16
-12
-8
-4
0
4
8
12
16
20
24
HOURS AFTER ara-C INJECTION

Chart 18

declined to a low of 1.60% at 20 hours, and by 24 hours had attained the control level.

The mean DI data of this series are presented in Chart 16C. The peak in this experimental series was attained 8 hours after the last ara-C injection, 17.47%. From then on, the DI declined steadily to 2.37% in the last group.

Section III

This section describes the observations on the effect of ara-C on the cell cycle, asynchronous and synchronized cell populations of the B16 melanoma.

To ascertain the effect of ara-C on the cell cycle of B16 melanoma on day 6 of tumor growth, a single 50 mg/kg dose was administered to 56 tumor bearing mice at 9 p.m. on day 5 of tumor growth. Twelve hours later a single injection of H^3-thymidine, 50μC/0.25 ml in distilled water, was administered to these animals. They were subdivided into 14 groups. The first group was killed 1 hour after having received the pulse label. The remaining 13 groups were killed at 2 hour intervals over the next 26 hours. The percentage labeled mitoses of the individual tumors and the group means, are listed in Table 19, and graphically illustrated in Chart 19A. From these data, the t_C of B16 melanoma on day 6 of tumor growth, following a single 50 mg/kg i.p. dose of ara-C, was estimated to be 12 hours. The duration of S was determined to be 9.0 hours while $G_2 + \frac{1}{2}M$ equalled 2.0 hours. From these determined durations: $G_1 + \frac{1}{2}M = t_C - (S + G_2 + \frac{1}{2}M) = 1$ hour.

TABLE 19

PERCENTAGE LABELED MITOSES OF DAY 6 B16 MELANOMA
12 HOURS AFTER ARA-C ADMINISTRATION[a]

Hours After H^3-Thymidine Injection	Percentage of Labeled Mitoses[b]				Mean ± S.D.
1	15	12	9		12.00 ± 2.45
3	68	85	88		80.33 ± 8.84
5	76	77	89	80	80.50 ± 5.12
7	82	88	90	97	89.25 ± 5.36
9	83	94	91	88	89.00 ± 4.06
11	42	70	51	38	50.25 ± 12.34
13	63	56	41	39	49.75 ± 10.08
15	75	57	56		62.67 ± 8.71
17	60	62	72	57	62.75 ± 5.63
19	53	44	45	37	44.75 ± 5.67
21	43	76	37	35	47.75 ± 16.57
23	48	71	40	56	53.75 ± 11.45
25	25	36	52		37.66 ± 14.61
27	44	27	48	51	42.50 ± 9.29

[a]data plotted in Chart 19a.

[b]each figure represents a single tumor.

Chart 19.

Labeled mitoses curves of B16 melanoma 12 hours after a single 50 mg/kg injection of ara-C. A. On day 6 of tumor growth, and B. on day 18 of tumor growth. The groups were killed at 2 hour intervals after a pulse label of H^3-thymidine. Each point represents the data from a single tumor. The solid lines are drawn through the means of each group.

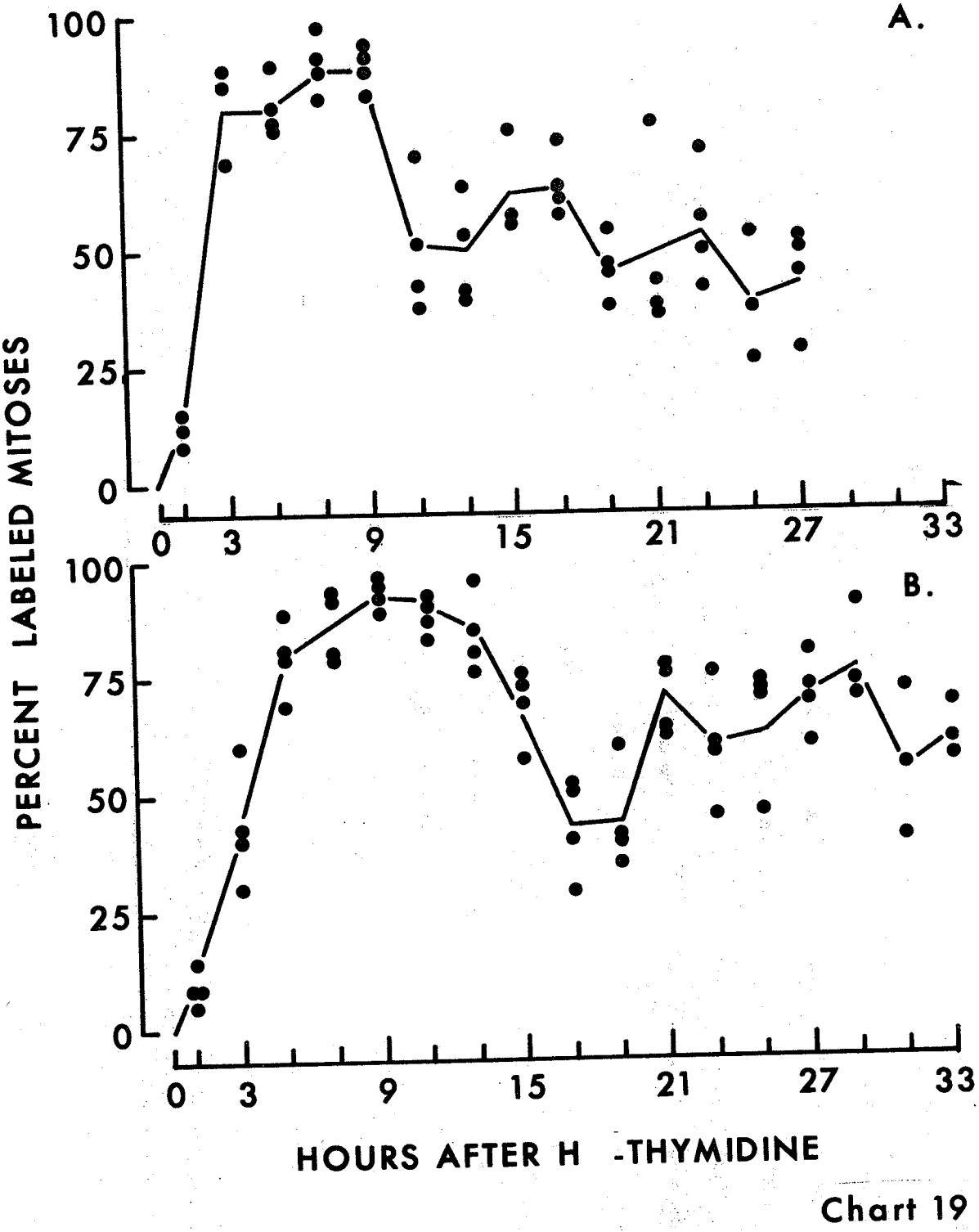

A.
B.
100
75
50
25
0
0
3
9
15
21
27
33
PERCENT LABELED MITOSES
HOURS AFTER H -THYMIDINE

Chart 19

The effect of the identical dosage ara-C on the cell cycle of B16 melanoma on day 18 of tumor growth was determined with 65 tumor bearing mice. The drug was administered at 9 p.m. on day 17 of tumor growth. Twelve hours after the last injection of ara-C, the animals received the H^3-thymidine pulse label. They were divided into 17 groups, the first 14 composed of 4 mice, the last 3 groups of 3 mice each. The first group of mice was again killed 1 hour after the pulse label, the remaining 16 groups at 2 hour intervals over the next 32 hours. The percentage labeled mitoses of the individual tumors, together with the group means are listed in Table 20 and plotted in Chart 19B. The t_C of B16 melanoma on day 18 of tumor growth, following a single 50 mg/kg ara-C dosage was estimated to be 20 hours. The duration of the S phase was determined to be 12.5 hours, while $G_2 + \frac{1}{2}M$ equalled 3.5 hours. It became evident from those durations that: $G_1 + \frac{1}{2}M = t_C - (S + G_2 + \frac{1}{2}M) = 4$ hours.

The effect of a single 40, 50 or 60 mg/kg dose of ara-C on the LI_{15}, MI and DI of asynchronous tumors on the 10th day of tumor growth. This information was determined by employing 84 tumor bearing mice. Twenty-eight mice were subdivided into 7 groups, and each received a single i.p. injection of 40 mg/kg of ara-C at 10 a.m. on day 10 of tumor growth. The first group was killed 1 hour after a 15 minute pulse label of H^3-thymidine. The remaining 6 groups were killed at 4 hour intervals over the next 24 hours, namely at 3 p.m., 7 p.m., 11 p.m., 3 a.m., 7 a.m., and 11 a.m. The identical procedure was applied to the remaining 56 mice, with half of them receiving a single 50 mg/kg, the other a 60 mg/kg injection of ara-C. The mean LI_{15}, MI and DI data of the 3 experimental groups are listed in Table 21 and illustrated in

TABLE 20

PERCENTAGE LABELED MITOSES OF DAY 18 B16 MELANOMA
12 HOURS AFTER ARA-C ADMINISTRATION[a]

Hours After H^3-Thymidine Injection	Percentage of Labeled Mitoses[b]				Mean ± S.D.
1	15	9	5	9	9.50 ± 3.64
3	60	30	41	43	43.50 ± 10.74
5	80	81	69	88	79.50 ± 6.80
7	93	81	79	94	86.75 ± 6.80
9	89	96	95	92	93.00 ± 2.74
11	90	92	88	83	90.75 ± 1.92
13	96	80	76	85	84.25 ± 7.50
15	57	72	69	75	68.25 ± 6.83
17	52	41	51	29	43.25 ± 9.28
19	41	60	40	35	44.00 ± 9.51
21	76	63	77	65	70.50 ± 2.12
23	45	76	60	59	60.00 ± 10.98
25	46	71	73	72	63.00 ± 21.18
27	60	69	80	73	70.50 ± 7.23
29	90	74	71		78.33 ± 8.37
31	72	40	55		55.66 ± 13.10
33	68	60	57		61.66 ± 4.73

[a]data plotted in Chart 19b.

[b]each figure represents a single tumor.

TABLE 21

LABELING INDEX, MITOTIC INDEX AND DEGENERATING INDEX FOLLOWING A SINGLE 40, 50 OR 60 mg/kg INJECTIONS OF ARA-C

Dose of Ara-C (mg/kg)	Hours After Ara-C Injection	Number of Tumors	Labeling[a] Index (mean ± S.D.)	Mitotic[a] Index (mean ± S.D.)	Degenerating[a] Index (mean ± S.D.)
40	1	4	1.23 ± 0.28	1.35 ± 0.30	1.54 ± 0.57
	5	4	2.37 ± 0.73	0.10 ± 0.17	1.90 ± 0.84
	9	4	21.27 ± 12.61	0.19 ± 0.33	2.18 ± 0.73
	13	4	31.03 ± 6.52	0.87 ± 0.80	1.87 ± 0.61
	17	4	11.74 ± 5.86	1.32 ± 0.37	4.03 ± 2.52
	21	4	22.25 ± 12.79	2.22 ± 0.57	2.63 ± 1.24
	25	4	25.40 ± 7.68	2.25 ± 0.53	2.24 ± 0.57
50	1	4	0.42 ± 0.36	2.95 ± 1.03	1.92 ± 0.53
	5	4	1.36 ± 0.62	0.25 ± 0.20	1.53 ± 0.14
	9	4	13.69 ± 5.79	0.41 ± 0.45	1.84 ± 0.48
	13	4	17.16 ± 5.27	2.42 ± 0.88	2.14 ± 0.42
	17	4	18.94 ± 4.92	3.00 ± 0.80	2.00 ± 0.56
	21	4	18.31 ± 4.81	2.06 ± 0.39	1.61 ± 0.82
	25	4	17.43 ± 4.96	2.37 ± 0.91	2.19 ± 0.82
60	1	4	1.93 ± 0.84	1.84 ± 0.71	1.64 ± 0.84
	5	3	2.34 ± 0.62	0.13 ± 0.00	1.59 ± 0.44
	9	4	2.88 ± 1.33	0.30 ± 0.41	2.10 ± 0.66
	13	4	24.83 ± 7.76	2.30 ± 1.57	2.50 ± 0.24
	17	4	14.06 ± 3.46	1.96 ± 0.99	2.43 ± 1.21
	21	4	9.19 ± 2.63	2.54 ± 1.14	4.25 ± 0.88
	25	4	21.83 ± 4.66	2.55 ± 0.92	2.77 ± 1.09

[a]expressed as a percentage.

Chart 20.

The LI_{15} values of all three series were roughly similar at the one and five-hour intervals following ara-C administration, although the LI_{15} was somewhat higher in the five-hour groups than in the one-hour groups. The LI_{15} of the series that received a 40 or 50 mg/kg ara-C dosage had increased by 9 hours to 21.27% and 13.69%, respectively. In the 60 mg/kg series, there was only a slight subsequent increase from the 5 hour group, to 2.88%. The LI_{15} of the series receiving a 40 or 60 mg/kg ara-C reached the peak after 13 hours, 31.03% and 24.83%, respectively. In the 50 mg/kg series, the LI_{15} increased further to 17.16% after the 13 hours. There was a significant difference between the LI_{15} of the 40 mg/kg ($p < 0.05$) and 50 mg/kg groups, but not so of the 60 mg/kg groups. The LI_{15} of the 50 mg/kg group attained the maximum of 18.94% at 17 hours after the ara-C injection. By that time the LI_{15} of the 40 and 60 mg/kg groups had declined to 11.74 and 14.06%, respectively. At 21 hours, the LI_{15} data of the 60 mg/kg series was significantly different ($p < 0.05$) from both the LI_{15} of the 40 and 50 mg/kg series. By the time the final time interval was reached, the LI_{15} of the 40 and 60 mg/kg series had increased above the control LI_{15} level of 18.6%, whereas the LI_{15} of the 50 mg/kg series declined further below the control level to 17.43%.

With all dosages, the one hour MI remained unchanged from the 2.18% control level; by five hours the MI data of all series were below the 0.26% level. In the 9-hour groups the MI remained near the zero mark in all series, but by 13 hours had regained the control level in the 50 and 60 mg/kg series. In contrast, the MI of the 13 hour group of the 40 mg/kg series had increased to 0.87%. There was no statistically

Chart 20.

The mitotic index, degenerating index and labeling index of B16 melanoma after a single 40 (△), 50 (■) or 60 mg/kg (▲) injection of ara-C on the 10th day of tumor growth.

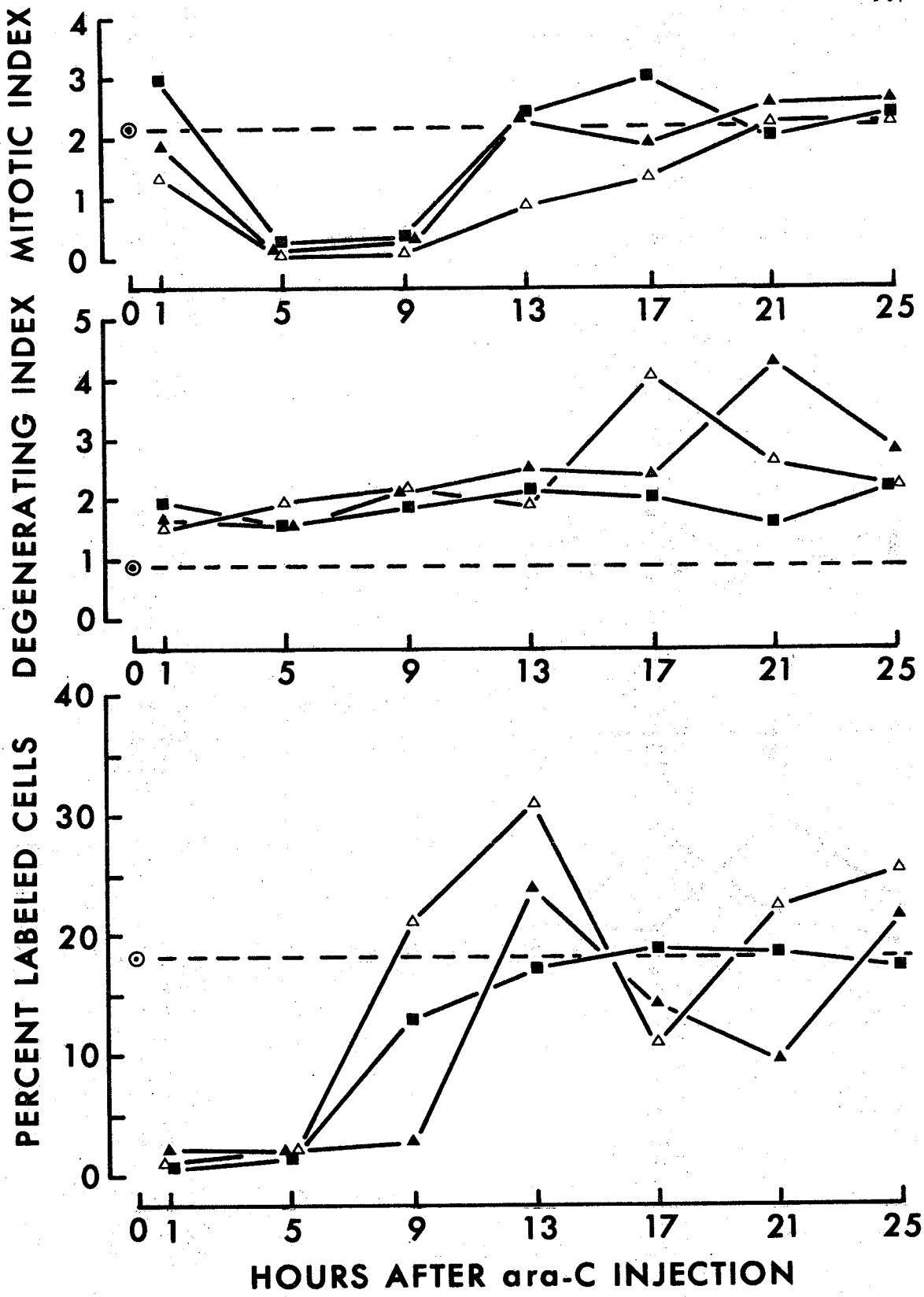

MITOTIC INDEX
4
3
2
1
0
0 1 5 9 13 17 21 25
DEGENERATING INDEX
5
4
3
2
1
0
0 1 5 9 13 17 21 25
PERCENT LABELED CELLS
40
30
20
10
0
0 1 5 9 13 17 21 25
HOURS AFTER ara-C INJECTION

Chart 20

significant difference between the 13 hour groups, however ($p > 0.05$). The MI values of the 17 hour groups, scattered about the control level, were also statistically the same ($p > 0.05$). Those of the last two groups were slightly above the control level.

One hour after the administration of the different ara-C doses, the DI data were slightly above the control DI level of 0.91%. They gradually increased in the subsequent groups. The DI of the 17 hour group in the 40 mg/kg series was 4.03%, but not significantly different from the same groups of the 50 and 60 mg/kg series. The DI of the 21 hour group in the 60 mg/kg series was 4.25%, and differed significantly from that group of the 50 mg/kg series ($p < 0.05$) but not the group of the 40 mg/kg series. The DI values of the last groups (25 hours) of all series were the same statistically.

The effect of three 40, 50 or 60 mg/kg dose of ara-C on the LI_{15}, MI and DI of asynchronous tumors on the 10th day of tumor growth. Eighty-four tumor bearing mice were imployed in these series. One third or twenty-eight of these received three single i.p. injections of 40 mg/kg of ara-C at 9 a.m., 10 a.m., and 11 a.m. on the 10th day of tumor growth. The first group of mice, after having received a 15 minute pulse label, was killed 1 hour later (12 noon). The remaining 6 groups were killed at 4 hour intervals over the next 24 hours, namely at 4 p.m., 8 p.m., 12 midnight, 4 a.m., 8 a.m. and 12 noon. The remaining 56 tumor bearing mice were divided into two further sub-groups receiving 50 or 60 mg/kg of ara-C at the same schedule of injections. The mean LI_{15}, MI and DI data of these 3 experimental groups are listed in Table 22 and illustrated in Chart 21.

TABLE 22

LABELING INDEX, MITOTIC INDEX AND DEGENERATING INDEX FOLLOWING THREE INJECTIONS OF 40, 50 OR 60 mg/kg INJECTIONS OF ARA-C

Dose of Ara-C (mg/kg)	Hours After Ara-C Injection	Number of Tumors	Labeling[a] Index (mean ± S.D.)	Mitotic[a] Index (mean ± S.D.)	Degenerating[a] Index (mean ± S.D.)
40	1	4	0.68 ± 0.20	0.05 ± 0.10	1.43 ± 0.40
	5	4	2.13 ± 1.66	0.00 ± 0.00	2.56 ± 1.33
	9	4	20.47 ± 2.65	0.22 ± 0.26	1.55 ± 0.41
	13	4	27.09 ± 4.83	0.00 ± 0.00	1.41 ± 0.28
	17	4	15.67 ± 6.81	1.95 ± 1.09	2.60 ± 0.79
	21	4	8.66 ± 2.94	1.91 ± 1.31	2.25 ± 0.60
	25	4	13.01 ± 3.98	3.08 ± 0.82	3.47 ± 1.35
50	1	4	0.97 ± 0.39	0.00 ± 0.00	1.51 ± 0.91
	5	4	5.80 ± 3.24	0.08 ± 0.10	2.32 ± 0.99
	9	4	11.12 ± 4.46	0.26 ± 0.17	2.45 ± 0.85
	13	4	21.45 ± 6.01	0.28 ± 0.28	2.27 ± 1.08
	17	4	4.81 ± 2.19	4.91 ± 1.57	2.39 ± 1.02
	21	4	7.16 ± 2.24	4.11 ± 2.05	2.26 ± 0.65
	25	4	13.27 ± 4.24	1.61 ± 0.41	2.03 ± 0.62
60	1	4	1.45 ± 0.30	0.08 ± 0.00	1.81 ± 0.87
	5	4	4.97 ± 2.13	0.34 ± 0.35	3.82 ± 1.34
	9	4	16.15 ± 11.95	1.03 ± 1.29	1.79 ± 0.48
	13	4	18.32 ± 4.83	0.92 ± 0.59	2.58 ± 0.52
	17	4	17.95 ± 7.06	4.35 ± 2.23	1.82 ± 0.61
	21	4	12.46 ± 3.79	2.14 ± 0.32	2.07 ± 0.66
	25	4	9.28 ± 3.97	2.09 ± 0.94	3.19 ± 1.56

[a]expressed as a percentage.

Chart 21.

The mitotic, degenerating and labeling index of B16 melanoma after three 40 (△), 50 (■) or 60 mg/kg (▲) injections of ara-C on the 10th day of tumor growth.

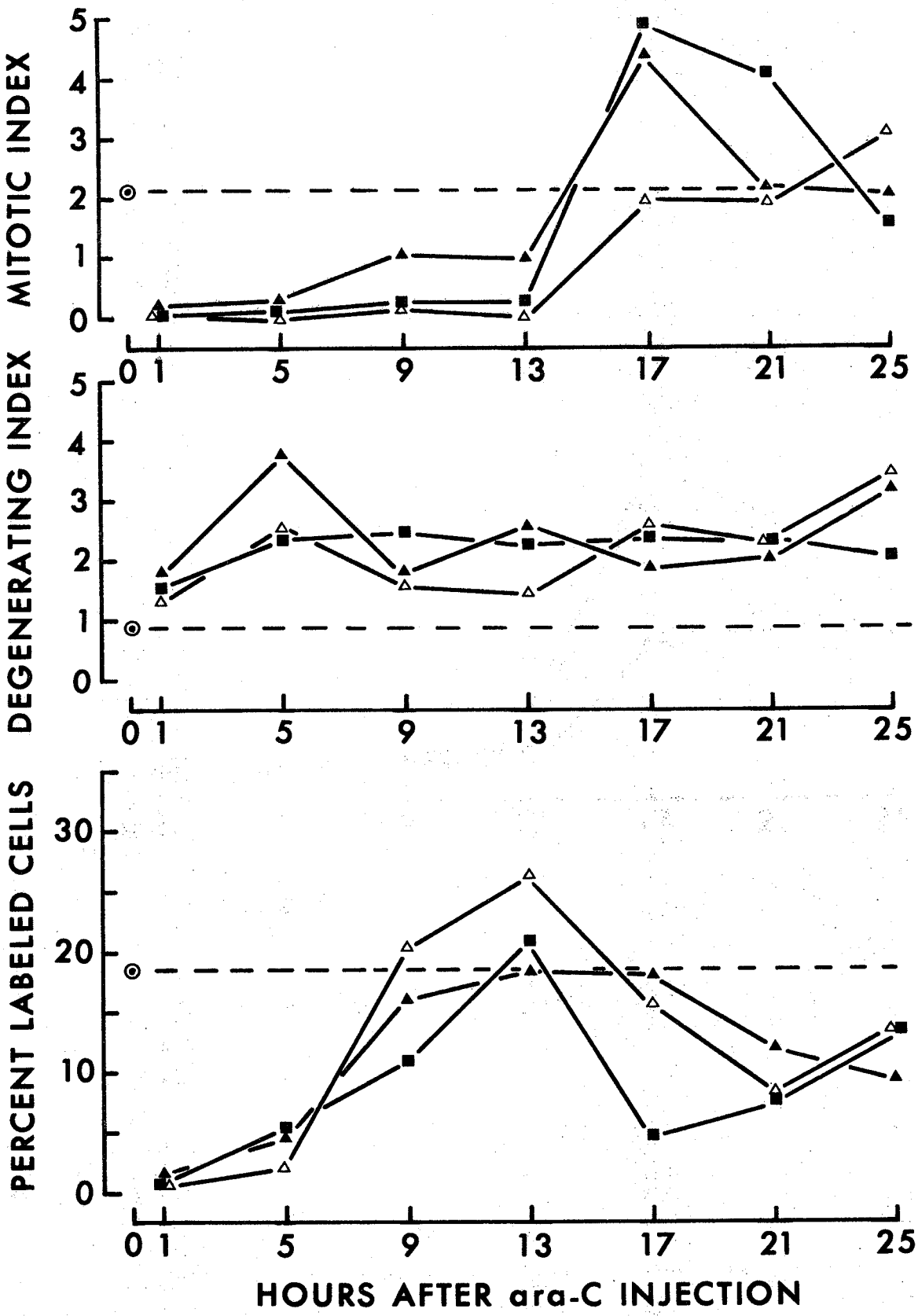

MITOTIC INDEX
DEGENERATING INDEX
PERCENT LABELED CELLS
0 1 5 9 13 17 21 25
HOURS AFTER ara-C INJECTION

Chart 21

One hour after the termination of the ara-C treatment, the LI_{15} values were relatively low, less than 1.46% in all three series. The LI_{15} increased subsequently. By 9 hours the LI_{15} of the 40 mg/kg series (20.47%) was significantly higher ($p < 0.05$) than the LI_{15} of the 50 mg/kg group (11.12%), but not so of the 60 mg/kg group (16.15%). The maximum LI_{15} was attained in all three series by 13 hours, and these were not statistically significant. Following the peak, the LI_{15} of the 17 and 21 hour groups declined, eventually reaching a low of 9.28% in the 60 mg/kg series and of 13.01% and 13.27% in the 40 and 50 mg/kg series by 25 hours.

The MI remained low in all three series until 9 hours following the ara-C injections. It subsequently rose to 1.03% in the 60 mg/kg series, whereas the MI of the other two series remained near the zero level until the 13 hour mark. By 17 hours the MI of all three series had become augmented to 1.95, 4.91 and 4.35% in the 40, 50 and 60 mg/kg series, respectively. The MI of the 50 mg/kg series differed significantly from the MI of the 40 mg/kg series ($p < 0.05$) but not so from the MI of the 60 mg/kg series ($p > 0.05$). In the last two groups, the MI of the 50 and 60 mg/kg series declined while it remained on the same level in the 21 hour group, and increased to 3.08% by 25 hours in the 40 mg/kg series. At that time the MI of the 40 mg/kg group differed significantly ($p < 0.05$) from the MI of 50 mg/kg group but not from the MI at 24 hours of the 60 mg/kg group.

The DI data of all three series one hour after the last ara-C injection were below 1.82%. The DI of tumors treated with 40, 50 and 60 mg/kg of ara-C were at the 1.43, 1.51 and 1.81% levels, respectively. The DI values of the three series at the subsequent four time intervals

remained below the 2.6% level in all cases, except the 5 hour group of the 60 mg/kg series which was 3.82%. The DI of the 25 hour groups were 3.47%, 2.03% and 3.19% in the 40, 50 and 60 mg/kg series, respectively. The latter were not significantly different.

The effect of a single 60 mg/kg dose of ara-C on the LI, MI and DI of a synchronized B16 melanoma tumor cell population. Sixty B16 melanoma bearing mice received 8 injections of ara-C (12.5 mg/kg) at 2 hour intervals (identical to series 4, Part II) to produce cell synchrony. Ara-C was administered at 10 p.m., 12 midnight, 2 a.m., 4 a.m., 6 a.m., 10 a.m., 12 noon and 2 p.m. on the 10th day of tumor growth. Ten hours after the last injection, in the synchrony procedure, a single 60 mg/kg dose of ara-C was administered to 56 tumor bearing mice with the cell population thus synchronized. At that 10 hour point, as ascertained in the previous series (Chart 14), the highest percentage of labeled cells was present. A group of 4 mice with a high degree of tumor cell synchrony, served as the marker group. The LI_{15} of that group was 40.44% (Table 23) as compared with the 10 hour group of the first series of synchronized population (Table 15) where it was 45.06%. Statistically both data were similar ($p > 0.05$). The LI, MI and DI values of the synchronized population, following the single 60 mg/kg injection of ara-C, are listed in Table 23 and illustrated in Charts 22 and 23.

The LI_{15} of the 2 hour group was 5.07%. This constituted a substantial decline from the 40.44% LI_{15} synchrony level of the marker group. By four hours the LI_{15} had further declined to 3.59%. The LI_{15} of subsequent groups declined but then rose again to a second peak of 18.90% by 20 hours. This second peak was followed by a decline of the LI_{15} (Chart 22), reaching a low of 5.41%, 28 hours after ara-C.

TABLE 23

LABELING INDEX, MITOTIC INDEX AND DEGENERATING INDEX OF B16 MELANOMA AFTER A SINGLE INJECTION OF ARA-C (60 mg/kg) 10 HOURS FOLLOWING THE SYNCHRONY PROCEDURE

Hours Following Synchrony	Number of Tumors	Labeling Index (mean ± S.D.)	Mitotic Index (mean ± S.D.)	Degenerating Index (mean ± S.D.)
0[a]	4	40.44 ± 5.06	0.57 ± 0.87	5.58 ± 1.84
2	4	5.07 ± 1.52	0.27 ± 0.33	5.72 ± 1.75
4	4	3.59 ± 0.32	0.46 ± 0.20	5.22 ± 0.49
6	4	13.54 ± 4.15	0.22 ± 0.37	4.70 ± 1.41
8	4	15.03 ± 3.33	1.23 ± 1.14	3.80 ± 0.67
10	4	17.41 ± 2.48	0.49 ± 0.42	5.46 ± 0.78
12	4	13.17 ± 3.63	2.95 ± 2.20	4.15 ± 1.50
14	4	9.70 ± 2.04	2.17 ± 0.84	3.71 ± 0.42
16	4	10.17 ± 1.86	1.45 ± 1.99	2.81 ± 1.17
18	4	12.37 ± 4.01	2.21 ± 0.70	3.37 ± 1.42
20	4	18.90 ± 0.88	1.96 ± 0.39	3.04 ± 0.39
22	4	10.36 ± 0.79	1.89 ± 0.75	4.09 ± 1.24
24	4	11.71 ± 2.58	1.77 ± 0.37	2.56 ± 0.59
26	4	10.89 ± 3.02	0.83 ± 0.28	2.54 ± 1.92
28	4	5.41 ± 1.63	2.77 ± 0.91	3.75 ± 0.59

[a]this group served as a marker as to the degree of synchrony achieved. This group did not receive the 60 mg/kg injection.

Chart 22.

Labeling and mitotic index of B16 melanoma after a single 60 mg/kg injection of ara-C 10 hours after cell synchrony. ⊙ represents the control asynchronous levels, ● the labeling index and ○ the mitotic index of the synchronized population. ▲△ marker groups of the synchronized population.

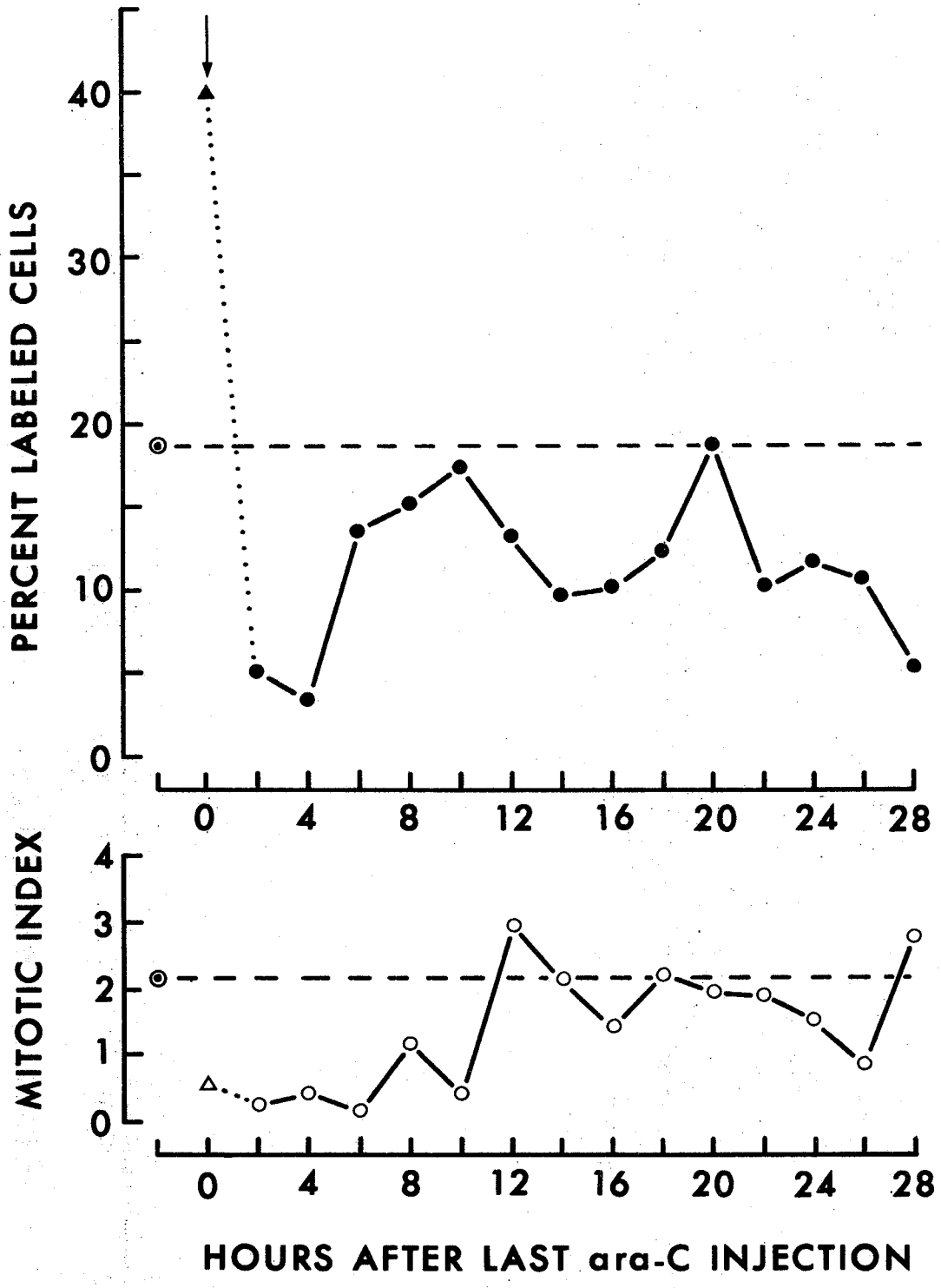

PERCENT LABELED CELLS
40
30
20
10
0
0
4
8
12
16
20
24
28
MITOTIC INDEX
4
3
2
1
0
0
4
8
12
16
20
24
28
HOURS AFTER LAST ara-C INJECTION

Chart 22

Chart 23.

Degenerating index of B16 melanoma after a single (△) and three (▲) 60 mg/kg injections of ara-C 10 hours after cell synchrony. ⊙ represents the control asynchronous level, ■□ the degenerating index of the synchronized population.

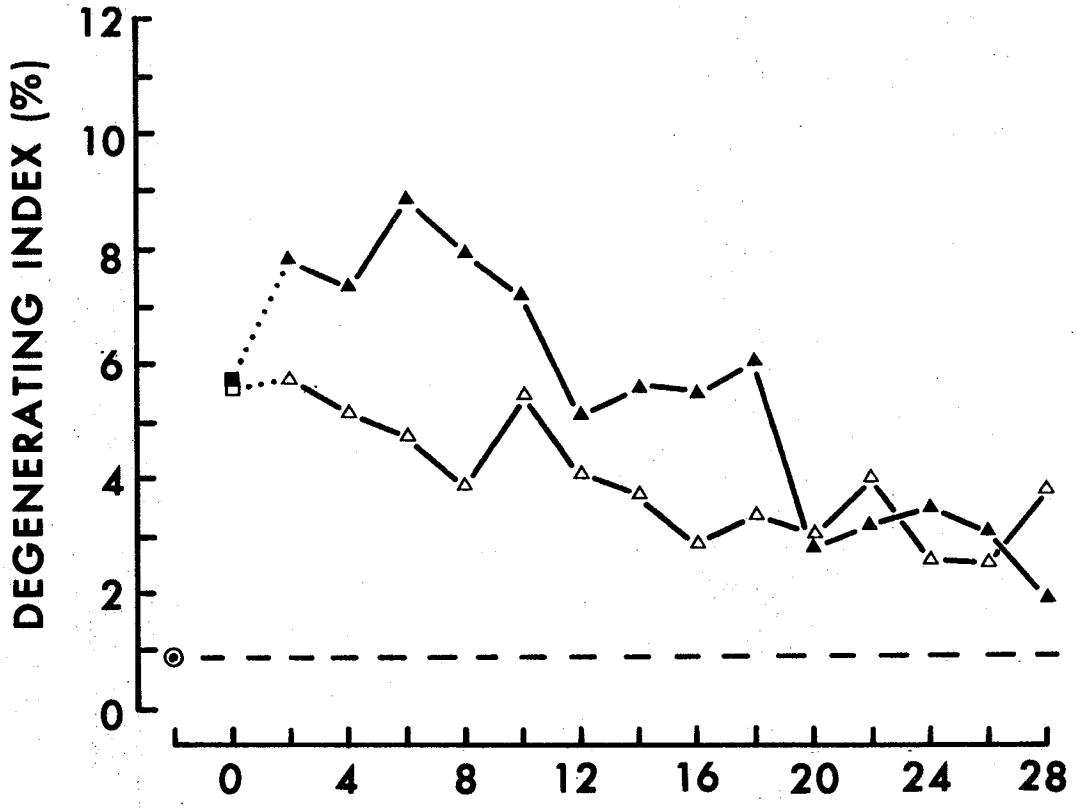

DEGENERATING INDEX (%)
12
10
8
6
4
2
0
0
4
8
12
16
20
24
28
HOURS AFTER LAST ara-C INJECTION

Chart 23

The MI of the first three groups (Chart 22) remained below 0.47%, that is lower than the 0.57% of the synchrony marker group. At this level, the MI values did not differ in any of the groups significantly. By 8 hours the MI had risen to 1.23% but declined subsequently to 0.49% by 10 hours. By 12 hours the MI had increased again to 2.95, but merely slightly above the asynchronous control level of 2.18%. The MI gradually declined in the subsequent groups to a low of 0.83% in the 26 hour group, although there was a slight augmentation despite this general trend at the 18 hour mark. The MI of the last group sampled at the 28 hours was 2.77%.

The DI of the synchrony marker was 5.58% which was statistically similar ($p > 0.05$) to the comparable group in the fourth series of the synchrony experiments. In general, the DI of the groups treated with a single 60 mg/kg dose of ara-C declined from the synchrony marker value (Chart 23). There were three divergent points at which the DI of the groups had increased, namely at 20, 26 and 38 hours.

Effect of three 60 mg/kg doses of ara-C on the LI, MI and DI of a synchronized B16 melanoma tumor population. Sixty B16 melanoma bearing mice received 8 injections of ara-C (12.5 mg/kg) at 2 hour intervals (as in Series 4 of Part II) to produce a synchronized cell population. Nine, ten and eleven hours after the last injection of ara-C, to bring about cell synchrony, three injections of 60 mg/kg ara-C were administered to 56 of the tumor bearing mice. The injections were timed with the highest percentage of labeled cells present (Chart 14) in the synchronized population. A group of 4 mice bearing the synchronized tumor cell population served as a marker group. The LI_{15} of the latter group (Table 24) was 39.52%, compared to 45.06% of the 10 hour group of the

TABLE 24

LABELING INDEX, MITOTIC INDEX AND DEGENERATING INDEX OF B16 MELANOMA AFTER 3 INJECTIONS OF ARA-C (60 mg/kg) 10 HOURS FOLLOWING THE SYNCHRONY PROCEDURE

Hours Following Synchrony	Number of Tumors	Labeling Index (mean ± S.D.)	Mitotic Index (mean ± S.D.)	Degenerating Index (mean ± S.D.)
0[a]	4	39.52 ± 7.21	0.91 ± 0.74	5.66 ± 1.85
2	4	1.99 ± 1.70	0.00 ± 0.00	7.77 ± 1.89
4	4	3.80 ± 2.17	0.00 ± 0.00	7.34 ± 2.07
6	4	7.99 ± 2.42	0.02 ± 0.00	8.71 ± 3.31
8	4	7.63 ± 1.39	0.00 ± 0.00	7.88 ± 0.30
10	4	11.62 ± 3.62	0.12 ± 0.14	7.26 ± 1.22
12	4	23.03 ± 2.69	0.33 ± 0.46	5.04 ± 0.84
14	4	14.39 ± 5.45	1.24 ± 0.81	5.66 ± 1.11
16	4	12.36 ± 1.49	0.00 ± 0.00	5.50 ± 0.77
18	4	7.62 ± 1.02	1.91 ± 0.57	6.07 ± 1.75
20	4	4.56 ± 2.96	2.07 ± 0.60	2.70 ± 0.56
22	4	6.82 ± 2.53	2.48 ± 0.97	3.18 ± 0.60
24	4	11.76 ± 2.18	1.65 ± 0.44	3.50 ± 1.26
26	4	11.84 ± 3.42	1.51 ± 0.77	3.10 ± 0.64
28	4	15.18 ± 4.84	1.38 ± 0.62	1.87 ± 0.35

[a]this group served as a marker as to the degree of synchrony achieved. This group did not receive the three ara-C injections.

Chart 24.

Labeling and mitotic index of B16 melanoma after three 60 mg/kg injections of ara-C 10 hours after cell synchrony. ⊙ represents the control asynchronous levels, ● the labeling index and O the mitotic index of the synchronized population. ▲△ marker groups of the synchronized population.

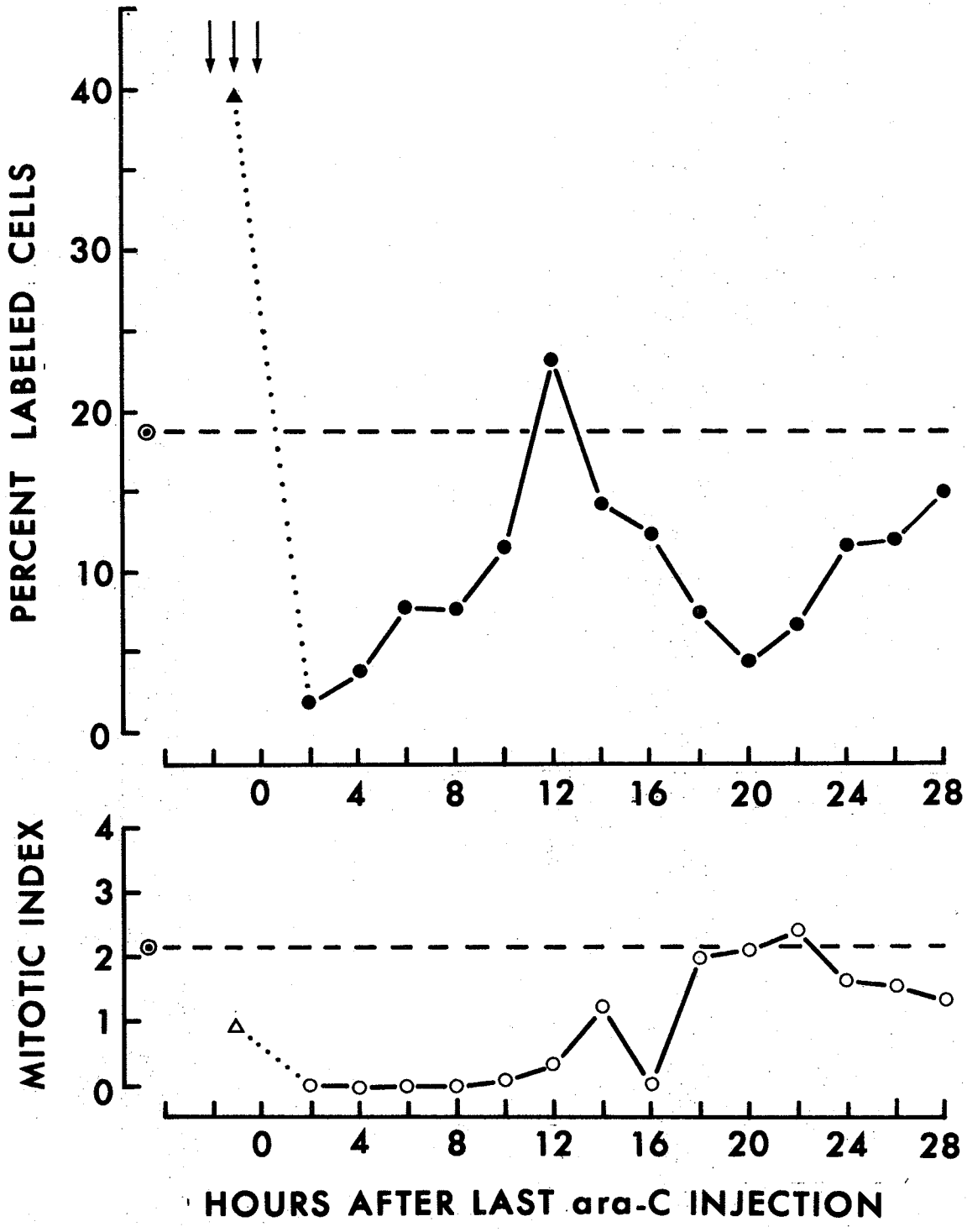

PERCENT LABELED CELLS
40
30
20
10
0
0
4
8
12
16
20
24
28
MITOTIC INDEX
4
3
2
1
0
0
4
8
12
16
20
24
28
HOURS AFTER LAST ara-C INJECTION

Chart 24

original synchronized population (Table 15); both were statistically the same ($p > 0.05$).

The LI, MI and DI data of the synchronized population following the three 60 mg/kg injections of ara-C, are listed in Table 24 and illustrated in Charts 23 and 24.

The LI_{15} of the group killed 2 hours after the last of 3 ara-C injections was 1.99%, well below the mean LI_{15} control level of 18.60%. The LI_{15} of the 4, 6, 8, 10 and 12 hour groups were 3.80, 7.99, 7.63, 11.62 and 23.03%, respectively. The 12 hour group attained a maximum surpassing the control LI_{15} level. The LI_{15} declined in the subsequent 6 groups to a low of 4.56% by 20 hours after the last ara-C injection. The LI_{15} of the remaining three groups was 11.76, 11.84 and 15.18% at 24, 26 and 28 hours after the last ara-C injection, respectively (Chart 24).

The MI of all first 5 groups were lower than 0.12%. It increased in the 6th and 7th groups to 0.33% and 1.24%, respectively (Chart 24). The MI of the synchrony marker group was 0.91. There was no significant difference between the MI of this group and that of the comparable group in Series 4 of the synchrony experiment. The MI of the 16 hour group declined to 0.00%. It became subsequently augmented in the 18, 20 and 22 hour groups to 1.91, 2.07 and 2.48%, respectively, and declined to 1.38% by 28 hours after the last ara-C injection.

The DI of the synchrony marker was 5.66%, statistically the same ($p > 0.05$) as the comparable DI of the group in Series 4 of the synchrony experiments. The DI values of the 2, 4 and 6 hour groups were 7.77, 7.34 and 8.71%. Following the peak at 6 hours the DI of the later groups declined to a low of 1.87% (Chart 23). Minor peaks

of DI occurred at the 18 and 24 hour periods after the last ara-C injection.

CHAPTER V

DISCUSSION OF RESULTS

The principal objectives of this investigation were: (1) to ascertain the cell kinetics of B16 melanoma so as to facilitate a more expedient interpretation of the *in vivo* cell synchrony study, (2) to achieve a high degree of cell synchrony in a malignant *in vivo* cell population with the use of cytosine arabinoside and (3) to study the effects of additional administrations of cytosine arabinoside on the previously synchronized tumor population.

A detailed study of the conventional cell cycle and kinetic parameters, as known for some solid tumors, have not so far been ascertained for B16 melanoma. Summary of the kinetic parameters of that tumor, as determined in the present study are listed in Table 25.

The mean survival time of mice bearing the highly malignant, transplantable B16 melanoma was 29.0 ± 6.9 days. The growth curves of that tumor displayed patterns very similar to other malignant tumors, for instance, the C3HBA mammary tumor (McCredie et al, 1965), mammary adenocarcinoma (Clifton and Yatvin, 1970), and C3H mammary carcinoma tumor cells in mice (Tannock, 1970). A similar curve was likewise observed for a melonic melanoma in the hamster (Simpson-Herron and Lloyd, 1970). Gross external measurements of the B16 melanoma tumor revealed that its growth was initially exponential but later decreased slowly at first and then more rapidly when approaching the terminal stage. Measurements beyond the mean survival time indicated that both the tumor area and mass became only slightly augmented. The mass doubling time (Table 2) became prolonged from 48 hours on day 6 of tumor growth, to 72, 120, 168 and 192 hours on days 10, 18, 25 and 33, respectively. That retardation with time of B16 melanoma growth, rather typical of solid tumors in general, was reflected also in the

TABLE 25

SUMMARY OF KINETIC AND CELL CYCLE DATA OF B16 MELANOMA ON VARIOUS DAYS OF TUMOR GROWTH

	DAYS OF TUMOR GROWTH								
	6	7	8	9	10	12	14	16	18
MI (%)	3.25	2.63	2.41	2.72	2.09 2.53 2.11 2.18[a]	2.04	2.56	2.33	1.32
DI (%)	0.75	0.79	1.08	1.22	1.04 0.77 0.79 0.91[a]	0.92	1.51	1.43	0.83
LI_{15} (%)					20.20 18.60[a]				
LI_{60} (%)	47.74				45.89 42.04				28.10
GF		0.57	0.47	0.57	0.49	0.55	0.50	0.59	
MR[b]	7.25				4.69				1.71
dMR (%)	87.00				56.30				20.50
dT (hrs)	27.60				42.60				117.00
Mass dT (hrs)	48.00				72.00				120.00
tC (hrs)	12.50								19.50
tG_2 + ½M (hrs)	1.50								3.50
tS (hrs)	9.50								15.50
tG_1 + ½M (hrs)	1.50								0.50
tM (hrs)	0.89								1.54
Total Labeled Continuous Exposure (72 hrs)					89.0%				

mean survival time = 29.0 ± 6.9 days.

[a] mean value for 16 groups (Table 11).

[b] represents the mitotic rate for a two hour interval.

declining mitotic and labeling indices. Thus the MI was 3.25% on the sixth day of tumor growth, declining subsequently to 2.09% on day 10 and 1.32% on day 18. Similarly, the LI_{60}, amounting to 45.89% on day 10 of tumor growth became reduced to 28.1% on the 18th day. Yet at an earlier stage of growth the LI_{60} remained rather stationary; it was 47.74% on day 6, and statistically similar to that on the 10th day (Table 25). All these indices signified the reduction of the proportion of cells within the mitotic and S phase compartments with progressing tumor growth.

The retardation of tumor growth was further reflected by the changes of the mitotic rate, determined by the colchicine technique. The two-hour mitotic rate on day 6 of tumor growth was 7.25%. It became reduced to 4.69% on day 10 and 1.71% on day 18 after tumor transplantation. Some questions may arise regarding the validity of a 2 hour mitotic rate as determined by the colchicine technique in this study, when comparing it to the daily mitotic rate on day 10 of tumor growth, ascertained previously by four 6 hour colchicine groups to be 34.0% (Bertalanffy and McAskill, 1964a); or by six 4 hour colchicine groups, yielding a figure of 46.1% (Bertalanffy and Gibson, 1971). The 2-hour groups seem to indicate a daily mitotic rate of about 56% (Table 3) which was higher than both previous estimates. However, these data of mitotic rate were merely intended for comparing approximate differences of daily mitotic rates between the days 6, 10 and 18 of tumor growth rather than to serve as absolute figures. In line with such comparisons the dMR declined from 87.0% on day 6 of tumor growth to 56.3% on day 10, and further to 20.5% on the 18th day. The tumor doubling time likewise declined with age, from 27.6 hours on day 6, over 42.6 hours on day 10,

to 117.0 hours on the 18th day after transplantation.

Perhaps one of the most decisive factors in the decline of the growth rate with age of the B16 melanoma was the circumstance that the mean cell cycle time of day 18 tumors became prolonged by 56% over that mean time of tumors in the 6th day after transplantation. The duration of the latter was 12.5 hours, with S equalling 9.5, G_2 + ½M, 1.5, and G_1 + ½M, 1.5 hours (Chart 9A).

In contrast, by the 18th day of tumor growth, the mean cell cycle time was extended to 19.5 hours, with S equalling 15.5, G_2 + ½M, 3.5, and G_1 + ½M, 0.5 hours (Chart 9B). The durations of S, G_2 + ½M and G_1 + ½M could be determined readily from the PLM curve of the day 6 tumors by the method of Quastler and Sherman (1959). However, some question arises as to the validity of the durations of the cell cycle components of the day 18 tumors. There appeared to be a break in the descending limb of the first PLM curve (Chart 9B) at 13 hours which was followed by a slower decline of the descending limb, indicated by a smaller slope. A smaller slope generally indicates a greater variation in the combined S + G_2 + M durations (Cleaver, 1967). The break in the descending limb may further imply a bimodal distribution of cell cycle time of the day 18 tumors. One cell population may have had a cell cycle duration similar to that of the day 6 tumors, while the cell cycle time of the second population may have been considerably longer. It was further possible that the mitotic figures in the day 18 tumors were a combined sample from a rapidly proliferating peripheral tumor zone and a slowly proliferating central region with different cell cycle times. A gradation of mitotic rates of that type in relation to the distance of a sample, extending from the central necrotic region

to the periphery, was observed previously in rat rhabdomyosarcoma (Hermens and Barendsen, 1969). Although all precautions were employed with the B16 melanoma to prevent such an occurrence, by sampling the tumors only from the periphery, it remains still a possibility that cannot readily be excluded. A third explanation of the break in the descending limb of the first PLM curve on the 18th day of tumor growth may be a unimodal distribution of cell cycle times with a longer duration altogether combined with a higher variability of the cycle times themselves. Whatsoever the reason, the data clearly indicates that there remained a significant proportion of cells in the 18 day tumors with cell cycle times longer than those on the 6th day of tumor growth. Previous studies on B16 melanoma (Bertalanffy and Gibson, 1971) revealed the duration of the cell cycle on day 10 to be 14 hours. That duration represented likewise a prolonged mean cell cycle time of 1.5 hours of the 10 day tumors over the 6 day tumors.

Other solid tumor populations that have been studied in this regard, have likewise revealed prolonged cell cycle times with age. Simpson-Herren and Lloyd (1970) observed that the cell cycle times increased with age in various solid rodent tumors: in adenocarcinoma 775, from day 4 - t_c = 12 hours to day 24 - t_c = 24 hours; Fortner plasmacytoma 1, from day 6 - t_c = 13.8 hours to day 19 - t_c = 16.5 hours; and sarcoma 180, from day 5 - t_c = 14 hours to day 8 - t_c = 26 hours. Thus also in the B16 melanoma, as it does in other solid tumors, the increase in the mean cell cycle time probably played a significant role in the retardation of tumor growth with age.

Another factor often involved in the retardation of tumor growth is a decline of the GF. A retarding GF was demonstrated in various tumors,

for instance, Ehrlich ascites tumor where the GF declined from 0.82 on the first day of tumor growth to 0.53 by the seventh day (Lala, 1968). Similarily in a solid murine fibrosarcoma the GF declined from 0.40 on the third to 0.24 on day 20 of tumor growth (Frindel et al, 1967). Rat adenosarcoma 755 and mouse sarcoma 180 revealed likewise a decline of the GF with age (Simpson-Herren and Lloyd, 1970). Yet, in the present study, it appeared that the GF of B16 melanoma (Table 25) did not decline between day 7 and 16 of tumor growth. The GF ranged from 0.47 to 0.59, with a mean of 0.53. Consequently, during the period the present experimental series were performed (days 7-16) the GF remained static and was not a decisive factor in the retardation of tumor growth. Mendelsohn (1962) likewise observed a static GF with age in a C3H mouse mammary tumor.

A further factor that may bring about a retardation of tumor growth is a quantitative change of cell loss from a tumor. The cell loss from B16 melanoma was not determined mathematically for any of the days of tumor growth. However, the DI was determined on different days of tumor growth. The DI (Table 25) increased slightly on days 14 and 16 but it was not statistically different from the DI on the other days. The DI was ascertained only in the viable peripheral regions of the tumor, and hence cannot be considered as an index of cell loss. A rough indication of cell loss was evident however, by the circumstance that during the early phases of tumor growth necrosis in the central tumor regions was minimal while it was extensive in older tumors, from the 14th day onward. This indirect evidence pointed to the likelyhood that cell loss by necrosis would play a significant role in the retardation of tumor growth particularily at later stages. Moreover,

Bertalanffy and McAskill (1964a) observed that 10 of 32 B16 melanoma bearing mice studied had secondary tumors from metastasis in other organs. Thus, cell loss by metastasis, as early as day 14 of tumor growth, participated also at least to some extent in the retardation of B16 melanoma growth with age.

It was evident from the experimental findings that the growth of B16 melanoma was rapid during the early stages but decelearted with time. It appeared that the principal factors in the retardation of tumor growth was a prolonged generation time and a loss of tumor cells chiefly by necrosis, with some metastasis conceivably participating. The GF itself did not decline between days 7 to 16 of tumor growth in B16 melanoma.

Previous studies by the author determined the duration of the cell cycle components and of the overall cell cycle of B16 melanoma on the 10th day of transplantation and Ehrlich ascites tumor on the 6th day (Gibson, 1969; Bertalanffy and Gibson, 1971). In the same report, it was evident that the S phase was shortened by the effect of a single 50 mg/kg dose of ara-C in B16 melanoma but not so in the Ehrlich ascites tumor. The total cell cycle time appeared to be prolonged by ara-C in B16 melanoma; whether such was also the case with the Ehrlich ascites tumor cells could not be determined from the experimental data. Chu and Fischer (1968a) likewise observed a lengthening of the generation cycle time of L5178Y cells after ara-C administration, and attributed it to cell death. The shortening of the S phase by ara-C in B16 melanoma may have been brought about by those cells that were in S at the time of ara-C administration, having accelerated DNA synthesis once they were released from the ara-C effect. Along these lines, Rajewsky (1970)

observed in a transplantable rat sarcoma that after cells were released from a hydroxyurea S phase block, they completed DNA synthesis and transversed G_2 at a faster rate than under normal conditions. In contrast, Karon and Shirakawa (1970) did not observe any change in the duration of G_2 in Don-C cells that had been treated with ara-C.

The cell cycles of B16 melanoma of ara-C-treated and untreated animals are compared on day 6 and day 18 in Table 26 and Chart 25. In the treated mice, the cell cycle times were determined 12 hours after a single injection of ara-C. This time interval was selected as by that time the LI_{15} had returned to the control level, following a single 50 mg/kg injection of ara-C (Bertalanffy and Gibson, 1971).

Comparing the two cell cycles and their subphases there appeared to have been a slight shortening of the S phase, but the overall duration of the cell cycle itself, remained unchanged. The only deviation between the means of comparable treated and untreated PLM curves was between the 19 hour groups ($p < 0.05$). Nonetheless, on the 6th day of tumor growth the slopes of the ascending and descending limbs of the first PLM curves were identical in both treated and untreated tumors. This signified that there was little, if any, variation in the length of the combined S + G_2 + M or G_2 + M phases (Cleaver, 1967). A pronounced effect (Chart 25) was the rapid fall of the percentage labeled mitoses in the ara-C treated cycle after 15 hours. The cell kinetic interpretation of this phenomenon, in terms of ara-C effect, is unknown.

A lengthening of the total cell cycle time was neither apparent on the 18th day; however, the S phase was markedly shortened. The latter lasted 15.5 hours in the untreated and 12.5 hours in the ara-C treated

TABLE 26

CELL CYCLE COMPONENTS OF UNTREATED AND ARA-C TREATED[a]

B16 MELANOMA ON DAY 6 AND 18 OF TUMOR GROWTH

Day of Tumor Growth	Dose of Ara-C	Cell Cycle Time (hours)	G_2 + ½M (hours)	S (hours)	G_1 + ½M (hours)
6[b]	0	12.5	1.5	9.5	1.5
6[c]	50 mg/kg	12.0	2.0	9.0	1.0
18[b]	0	19.5	3.5	15.5	0.5
18[c]	50 mg/kg	20.0	3.5	12.5	4.0

[a]12 hours after a single 50 mg/kg injection of ara-C.

[b]Chart 9.

[c]Chart 19.

Chart 25.

Untreated and ara-C treated PLM curves of B16 melanoma. A. Tumors on day 6 of tumor growth; B. tumors on day 18 of tumor growth. ▲, untreated PLM curves and △, ara-C treated PLM curves.

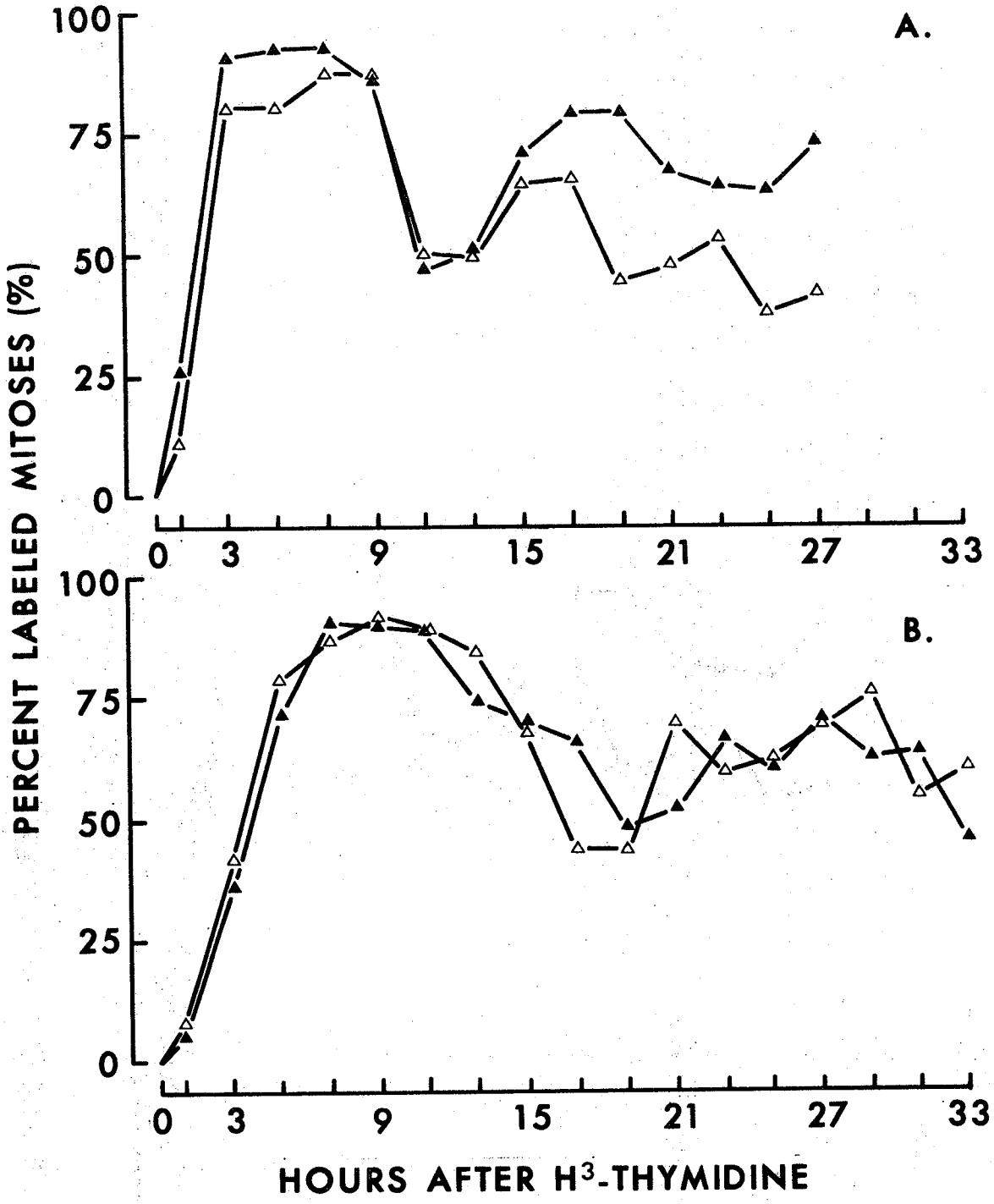

A.
B.
100
75
50
25
0
0 3 9 15 21 27 33
PERCENT LABELED MITOSES (%)
HOURS AFTER H3-THYMIDINE

Chart 25

tumors. The only deviation between the means of comparable treated and untreated PLM curves was in the 17 hour groups ($p < 0.05$). The ascending slopes of the treated and untreated PLM curves were similar, but the descending slope of the ara-C treated PLM curve was much greater, thus signifying a change in the variability of the $S + G_2 + M$ phases. It appeared therefore that 12 hours after a 50 mg/kg injection of ara-C the variation of the $S + G_2 + M$ phases became reduced in the day 18 tumors as compared to the untreated B16 melanoma. Similar observations were reported in vivo in L1210 leukemia on the 6th day of growth after an effective dose of 1,3-bis(2-chloroethyl)-1-nitorsourea (BCNU) and following retransplantation and growth (DeVita, 1971). In that series, the cell cycle data suggested a prolonged t_C and t_S, indicating either regrowth of surviving cells having altered their cycle times or else the selection of a cell line with a longer t_C duration.

It thus appears that the sole transient effect of ara-C on the cell cycle, 12 hours after its administration, in day 6 and 18 tumors, was a shortening of the S phase; this occurred to a greater extent in the day 18 tumors. However, in neither the day 6 of day 18 tumors was the total length of t_C altered. Ara-C probably selectively killed day 18 tumor cells, manifested by a reduced variability in G_2 - M segments of the cell cycle.

The primary aim of this investigation was the attempt to produce in vivo cell synchrony in a solid tumor, the B16 melanoma, utilizing the specific DNA inhibitor ara-C. The asynchronous B16 melanoma cell population did not reveal diurnal variation in the LI_{15}, MI or DI during the experimental period on day 10 (Table 11). Absence of diurnal variations in the LI_{15} or the MI, facilitated planning of the various

ara-C regimens. It was interesting to note the presence of a low DI in the viable areas of untreated B16 melanoma tumors indicating that individual tumor cells were dying.

Two factors were taken into account when planning the ara-C regimens: (1) the mean cell cycle time of 14 hours of the B16 melanoma, with a minimum S duration of 7.5 hours, and (2) that a single 50 mg/kg injection of ara-C reduced the LI to less than 1% for at least the subsequent 4 hours (Gibson, 1969; Bertalanffy and Gibson, 1971).

The seven regimens applied, in an attempt to produce in vivo cell synchrony, are presented in Table 27. The degree of synchrony was low following most of the ara-C regimen, with the highest SI equalling 40.7% in series 4. However, the GF of B16 melanoma proved to be 0.53, implying that only 53% of the tumor cell population was in the proliferating compartment. Evidence of a non-proliferating fraction was provided by the continuous H^3-thymidine labeling experiment (Chart 8), where after 72 hours of continuous labeling merely 89% of the tumor cells had incorporated the tracer. Supporting further the circumstance that only about half of the cells of the population were in the proliferating fraction was the observation that the highest LI achieved even with the most effective synchrony procedure (Series 4) was merely 45% (Table 27). It is thus important to note that only those cells that were part of the proliferating compartment could be synchronized by the ara-C procedure, in theory a maximum of 53%. Therefore, the experimentally observed SI in the various series could be adjusted in relation to the 53% GF; these adjusted figures are listed in Table 27. In the fourth series, that adjusted SI was 76.8%. This constituted indeed a fairly high degree of cell synchrony whether of an in vitro or in vivo cell popu-

TABLE 27

SUMMARY OF SYNCHRONY OBSERVATIONS

Series	Dosage of Ara-C	Number of Injections	Intervals Between Injections (hours)	Synchrony Index (SI)*	Adjusted SI* To Growth Fraction	Highest % Labeled Cells Obtained (Mean)
1	12.5	4	4	9.2	17.3	17
2	25.0	4	4	9.2	17.3	18
3	12.5	8	1	17.3	32.8	24
4	12.5	8	2	40.7	76.8	45
5	18.8	8	2	23.2	43.8	32
6	12.5	16	1	31.4	59.2	39
7	18.8	16	1	33.7	63.6	39

*Expressed as a percentage.

lation. The adjusted SI thusly yielded a certain overestimate because a number of cells in the proliferative fraction in the S phase at the time of ara-C administration, as in late G_1 in instances of a prolonged ara-C block were killed by the drug. A factor offsetting the cell kill in short range series would be the doubling of cells passing from mitosis into G_1. Hence, in the final reckoning it appears reasonable to assume that the adjusted SI would approach closer the real situation than the experimentally observed SI.

The two regimens yielding the best adjusted SI levels were those in series 4 and 7. The LI_{15} and MI curves of both series are plotted in Chart 26.

An interpretation of these curves is expedient in revealing the effects of an extended ara-C block on the proliferative cycle of the B16 melanoma cell population. It is well established that ara-C constitutes an inhibitor of DNA synthesis in mammalian cells. Specifically, ara-C prevents the synthesis of DNA as long as it remains at effectively high blood levels. Skipper et al, (1967) reported that the half-life of ara-C in the blood serum of mice was approximately 60-90 minutes. Although ara-C inhibits most cells engaged in DNA synthesis, it appears that cells in the latter 1-2 hours of the S phase remain unaffected by the drug. Conceivably the synthesis process may have progressed beyond a point where DNA synthesis cannot be inhibited any longer (Bertalanffy and Gibson, 1971). Most authors believed that the majority of cells in the S phase were blocked, while those in G_2, M or G_1 at the time of ara-C administration proceed through the cell cycle to the very end of G_1, or to the G_1-S junction, and were there prevented from entering S. Cells in the S phase are either halted in S by

Chart 26.

LI_{15} and MI curves of synchronized B16 melanoma. A, From series 4 and B. from series 7. ▲, LI_{15} curves; △, MI curves; and symbols on the y-axis represent the asynchronous control levels.

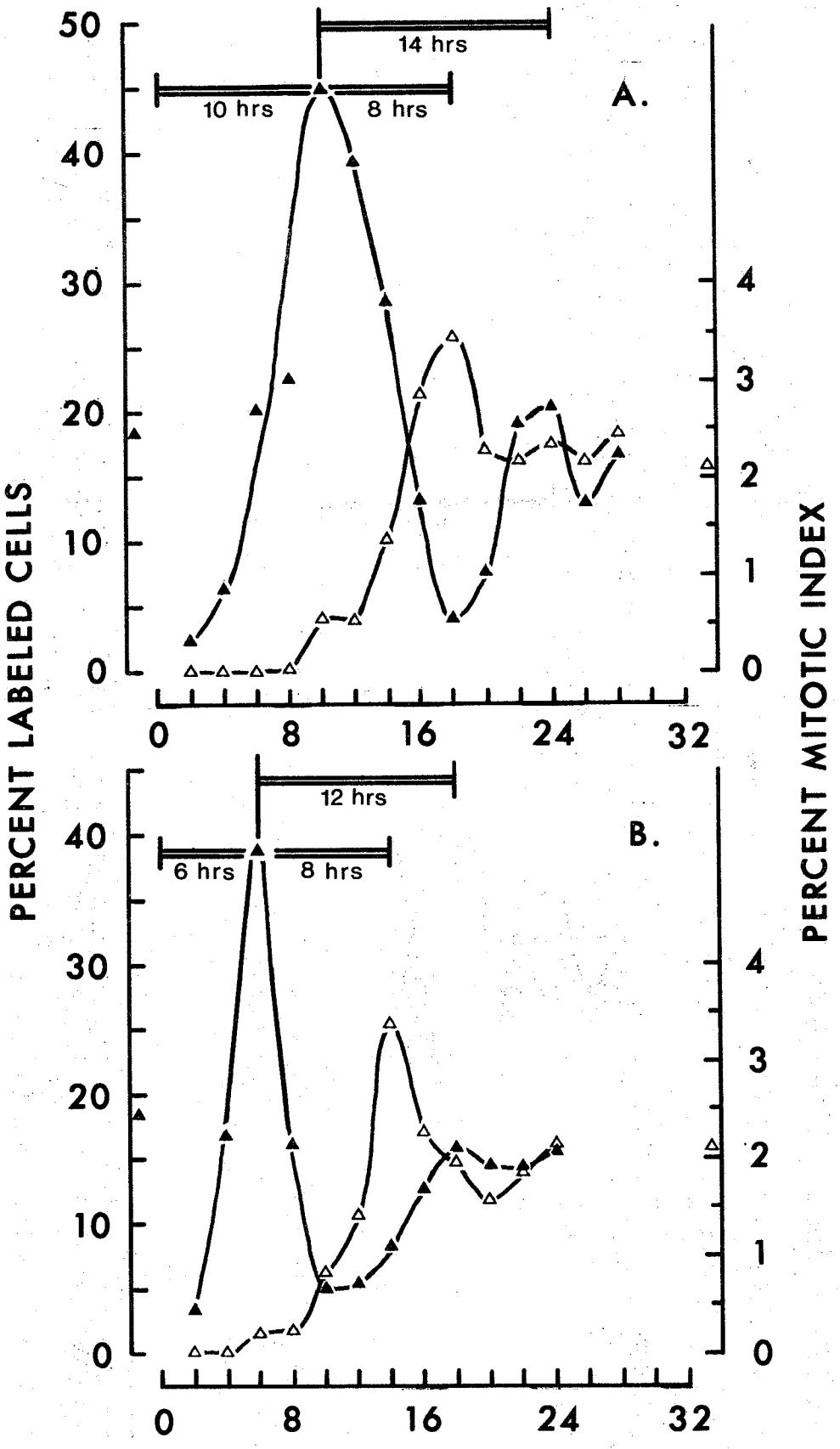

14 hrs
10 hrs
8 hrs
A.
50
40
30
20
10
0
4
3
2
1
0
0
8
16
24
32
12 hrs
6 hrs
8 hrs
B.
PERCENT LABELED CELLS
PERCENT MITOTIC INDEX
HOURS AFTER LAST ara-C INJECTION

Chart 26

a single injection of ara-C or else are killed outright. The amount of that cell kill in S appears to be dependent on both the dosage of ara-C and the length of the effectiveness of the drug in cases of multiple administrations.

Karon and Shirakawa (1969, 1970) reported that after ara-C treatment the transit time was greater at the S-G_2 junction than at the G_1-S junction and thus concluded that the cells were not blocked at the G_1-S junction. However, a significant amount of observed data leads one to believe that ara-C effectuates a block of cells at the G_1-S junction. Kim and Eidinoff (1965) concluded from cell kinetic criteria that HeLa S-3 cells were halted from entering S, and that cells not at the end of G_1 at the beginning of ara-C treatment accumulated with time at that point. Brent et al (1965) observed in synchronized HeLa cells that there was an augmented thymidine kinase activity in cells in the latter part of the G_1 phase. Kit et al, (1966) asserted on the basis of observations on HeLa cells inhibited by two different DNA inhibitors (ara-C and mitomycin) that an increase in thymidine kinase activity implied that the cells were blocked at the end of the G_1 phase. Bremerskov et al, (1970) likewise concluded from cytophotometric criteria that ara-C, as a cytostatic drug, produced selective killing of S phase cells and an accumulation of G_1 phase cells.

The effect of 16-hour blocks of ara-C (series 4 and 7) in the progression of B16 melanoma cells through the cell cycle as indicated by LI_{15} and MI curves are plotted in Chart 26. Two hours after the last ara-C injection few cells had incorporated H^3-thymidine. This implies that DNA synthesis of most cells remained still inhibited by ara-C. The LI_{15} curves attained their maximum in series 4, 10 hours

after the last ara-C injection, and 6 hours later in series 7. Both peaks of the LI_{15} were more than double that of the asynchronous control levels. Subsequent to these peaks the LI_{15} curves declined and then rose again to form second peaks 14 and 12 hours, in series 4 and 7, respectively. Each peak was assumed to represent the mid-point of the S phase of the cell cycles. Therefore, the time intervals between the first and second peaks were taken to equal the total cell cycle times following the two series of ara-C administration. As the cell cycle duration of B16 melanoma was determined to be 14 hours (Bertalanffy and Gibson, 1971) it appeared that in series 4 the ara-C regimen did not interfere with the progression of cells through the cell cycle after the release of the 16-hour block by ara-C. Similarly, it can be concluded that the regimen of ara-C in series 7 caused the released cells to pass through the cell cycle 2 hours faster than both series 4 and the asynchronous tumor cell population. The degree of synchrony attained in these two series became rapidly lost as it was evident from the low magnitude of the second peak of the LI_{15} curves. Such synchrony decay has been attributed to the independent variabilities of the cells comprising the entire proliferating cell population (Engelberg, 1964).

In the fourth series the MI curve was near the zero mark at 8 hours; it began to rise 10 hours after the release of cells from the G_1-S block. The members of an asynchronous population would normally pass through S + G_2 and into mitosis within (7.5 + 1.3 hours) 8.8 hours. It appeared therefore that the particular regimen of ara-C did not affect the t_c because the 1.2 hour difference could presumably be accounted for by variation. It signified at the same time that the block of S phase cells was complete. However, in series 7 the MI curve began to increase at 6 hours, that is, much earlier than would be

expected; this indicated that the block was either ineffective, or more likely that the cells released from the G_1 block transversed the S + G_2 phases 2-3 hours faster than normally. Such acceleration of the passage of cells through the S + G_2 phases would also explain the shortened cell cycle time evident from the LI_{15} curve. Rajewsky (1970) interpreted a similar observation of shortened cell cycle time to mean that the inactivation of S cells by ara-C was incomplete. In both series 4 and 7, as also in series 3 and 6, an 8 hour interval elapsed between the first LI_{15} peak and the first MI peak. The progression of cells through ½S + G_2 + ½M phases of the cell cycle would normally require 5.75 hours; thus, there appeared to be a discrepancy of 2.25 hours. This may be ascribable to the possibility that the duration of the S phase of B16 melanoma may be in fact longer than 7.5 hours, which was established as the minimum duration of S in the asynchronous cell population (Bertalanffy and Gibson, 1971). If the S phase was in fact 10.0 hours, not unreasonable considering that the S phase was normally 9.5 hours in day 6 tumors, then the duration of ½S + G_2 + ½M would be about 7 hours. That figure comes closer to the actually observed 8 hour duration between the LI_{15} and MI peaks. Another possibility may be that there was a small G_2 block as suggested by Karon and Shirakawa (1970) and Bremerskov et al, (1970). However, this possibility appears to be ruled out by the fact that the total cell cycle time was not prolonged after the ara-C regimen of series 4 and in fact was shortened by 2 hours following the ara-C regimen of series 7.

The DI of all series attained their maximum between 6 and 14 hours after the last ara-C injections; it then declined gradually towards the control level. It was thereby observed that the ara-C regimen

employed in the two series with the higher SI were also more effective in killing cells, as this is signified by their DI data, plotted in Chart 27. The first sampled groups following the last ara-C injection in series 4 and 7 displayed DI values of 2.26 and 6.72%, respectively and were well above the 0.91% of the asynchronous control DI. Interestingly enough, the DI peak occurred in both series 8 hours after the last ara-C injection. The relationship between a high SI and a high DI was not too clear. It might imply that the ara-C regimen in both series, 4 and 7, killed a greater proportion of cells in the S phase at the time when ara-C became effective than in the other series. This would further mean that on cessation of the ara-C effect fewer viable cells remained in S capable to proceed with DNA synthesis as a separate cohort apart from the much greater proportion blocked at the G_1-S junction. However, a distinct cohort of separately labeled cells was not observed. Therefore, it was more likely that the cell group blocked at the juncture of G_1-S for a period considerably exceeding the duration of S experienced some loss of viability. It would appear that such cells were unable to synthesize DNA as the synchronized cohort entering the S phase and thus died giving an increase in the DI 8 hours after the cessation of the ara-C administration. Kim et al, (1967) demonstrated in HeLa S-3 cells affected by hydroxyurea for brief periods exhibited an appreciable loss of cell viability, but this only in those cells that were in the DNA synthesis phase at the time of HU administration. Kim et al, (1968) observed further in the same cell population, a reduced survival rate of cells accumulated in the G_1-S transition phase when either HU or ara-C were administered for an interval exceeding the S phase duration. Inasmuch as the DI peak in

Chart 27.

DI curves of synchronized B16 melanoma. ○, curve of series 4; ●, curve of series 7; and ⊙, asynchronous control level.

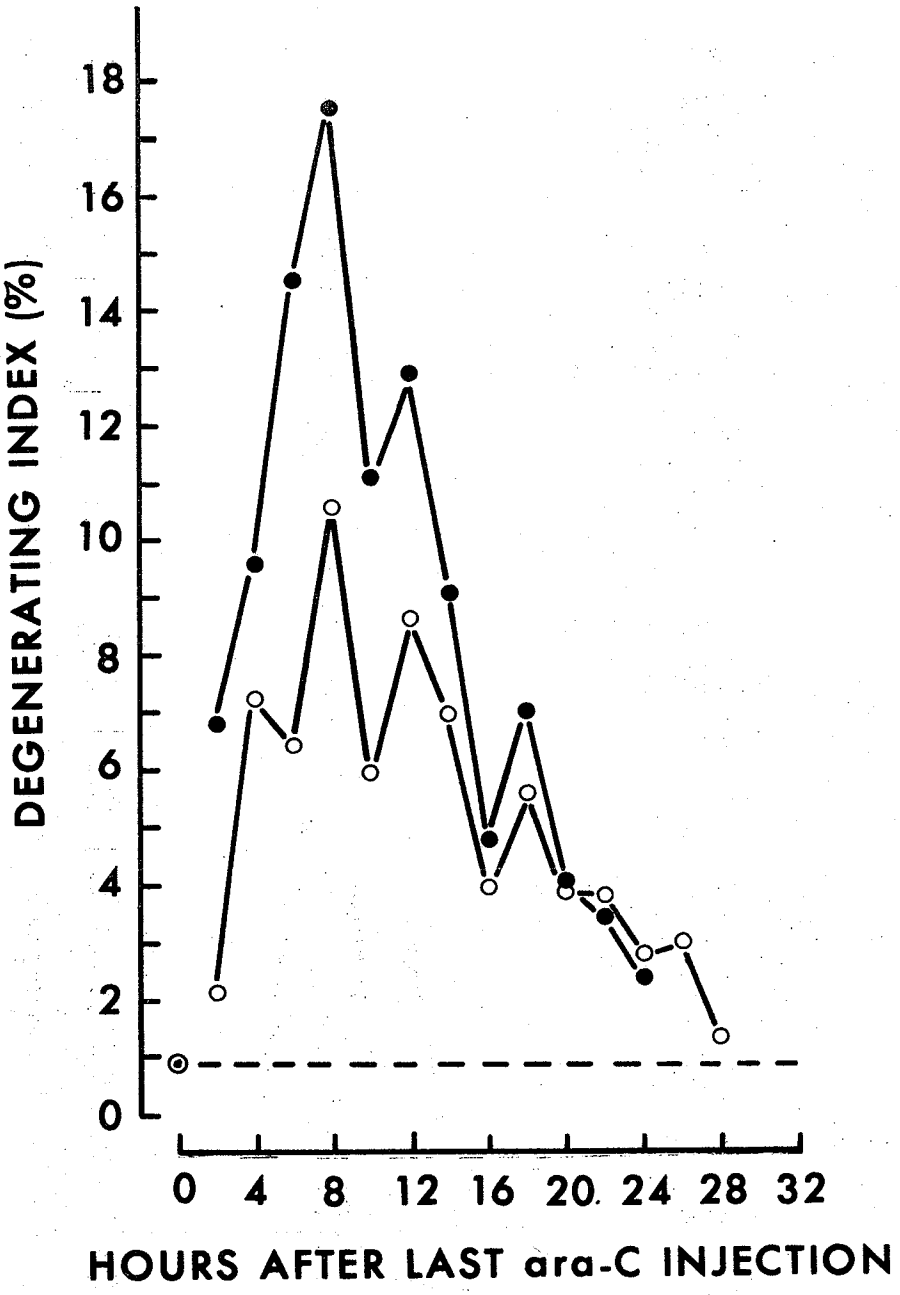

DEGENERATING INDEX (%)
18
16
14
12
10
8
6
4
2
0
0 4 8 12 16 20 24 28 32
HOURS AFTER LAST ara-C INJECTION

Chart 27

series 7 (Chart 27) exceeded that in series 4, the aforementioned factor might be the reason for the cell population exhibiting an SI lower than that observed in series 4, which revealed a lower DI peak. In other words, it appears that a larger number of G_1-S transition phase cells were killed by the ara-C regimen in series 7 than in series 4.

The DI curves (Chart 27) of these two series are identical in most aspects. The major difference was the magnitude of the DI peak in series 7. However, there were subsequent minor oscillations following the DI peak, such as at 12 and 18 hours after the last ara-C injection, the implication of which is unclear. It seems likely that the 18 hour peaks may be in line with the observations of Whitmore et al, (1969) demonstrating that there were two modes of cell kill by ara-C in mammalian cells. The first was in the S phase at the time the drug became effective and the other occurred after a time interval equal to S + G_2 + M. The exact reason for the cell kill in the second case remained undetermined. It seems reasonable to assume that this second period of cell kill affected those cells especially at the G_1-S junction, this was asserted by Kim et al, (1967, 1968).

All other ara-C regimen employed in the present study produced lower degrees of cell synchrony. It is believed that the low level of the SI achieved in both the first and second series was ascribable to the circumstance that four hour intervals between four successive ara-C injections failed to produce a sufficient block of cells synthesizing DNA. The DI of series 1 was lower while it was somewhat higher in the second series, probably related directly to the doubling of the ara-C dose. Yet, that same doubled ara-C dose failed to produce any

increase in the SI.

In series 3, where ara-C was administered hourly for eight consecutive hours, the SI was significantly augmented over those of the first two series; still, the adjusted SI was only 32.8 (Table 27). Theoretically, the 8 hour ara-C block should have been sufficiently long to allow the cells in the last 1-2 hours of the S phase, plus the G_2 + M and G_1 cells to proceed to the G_1-S junction, comprising a mean duration of 7.5-8.5 hours. Nonetheless, the peak of LI_{15} actually observed following the ara-C release was merely 23.62% or approximately one half of that in series 4. The reason for the low level of SI may be ascribable to variations in the duration of the different cell cycle phases (S, G_2, M and G_2) of individual cells, and hence not all of them did actually attain the end of G_1 by the 8 hours. This would be particularly true if the S phase of B16 melanoma was 9-10 hours instead of 7.5 as shown by Bertalanffy and Gibson (1970). Rajewsky (1970), employing a hydroxyurea block of comparable duration, produced a SI of 40-50% in a system with a GF of 0.9. His observations signify that the degree of SI achieved appears to depend on an adequate duration of the block which should exceed the total of the durations of the last segment of S + G_2 + M + G_1. The degree of cell kill in series 3 appeared likewise low suggesting that a proportion of cells in the S phase had actually survived the 8 hour ara-C block.

In series 5, the identical administration regimen was employed as in series 4, with the difference that ara-C dose was increased to 18.8 mg/kg. The peak level of DI attained was within 1% of that in series 4; however, the first two LI_{15} peaks were only 8 hours apart, possibly suggesting the presence of two synchronized cohorts of cells. Increasing

the dosage of ara-C in series 5 did not produce a higher SI than that in series 4. This may be attributable to yet unexplained factors observed by Mulligan and Mellett (1968), and Borsa et al, (1969), affecting the serum half-life of ara-C in mice. These authors demonstrated that the higher ara-C dosage of 250 mg/kg displayed a shorter half-life than the lower dosage of 50 mg/kg.

In series 6, the same regimen was used as in series 7, except that the dose of ara-C was lowered to 12.5 mg/kg from 18.8 mg/kg in series 7. The only plausible reason that can be offered for the higher SI in series 7 is again the aforementioned dosage level, for the total number of injections and the intervals between the ara-C administrations were identical in both series.

It is evident from all those observations that there was a combination of proper ara-C dosage, optimal intervals between the drug administrations and the proper number of injections which are crucial in the production of good cell synchrony. At the onset it appeared that the trial and error approach was the only one available for the determination of the proper dosage, intervals and number of injections of the drug for the production of cell synchrony in vivo. However, it soon became evident that there was a relationship between the degree of SI achieved and the magnitude of DI observed in any one regimen. If an excessive amount of cell kill occurred in a particular regimen, a large proportion of those cells at the G_1-S juncture were killed and the resulting SI was naturally low. This observation was particularly applicable to series 7. Another factor, obtained from the LI_{15} curves of most series, was that the decay of cell synchrony was rapid. This was obvious in those series were a reasonable amount of cell synchrony was

produced by the second LI_{15} peak always falling below the level of the first LI_{15} peak.

A principl effect of the DNA synthesis inhibition upon the cell kinetics of the *in vivo* tumor was a reduced LI_{15} persisting at least 5 hours after a single ara-C injection. One hour after the last ara-C injection, the MI remained still near the MI control level (Chart 20), because those cells that were in the latter part of S + G_2 remained unaffected by the drug and proceeded on to mitosis. However, by 4 hours the MI had declined and approached zero where it remained for a least the next 4 hours. This decline in the MI, a reflection of the absence of cells in mitosis, was attributable to the circumstance that those cells that were in the latter part of S and G_2 at the time of drug administration, had already passed through mitosis. The ara-C block inhibited cells from synthesizing DNA, thus preventing any flow of cells through G_2 into M. Both the LI_{15} and the MI recovered eventually, attaining a peak at 13 and 17 hours. Ara-C succeeded in roughly doubling the DI, from that of the asynchronous cell population, and the peak was attained at 17 and 21 hours in the experimental series receiving 40 and 60 mg/kg of ara-C. In general, the MI, LI_{15} and DI curve patterns were generally similar in all series, but exhibited also a few very obvious differences. The single 60 mg/kg ara-C dose was considered to be the most expedient from the chemotherapeutic viewpoint because DNA synthesis, as expressed by the LI_{15} was decreased to the lowest level and the greatest amount of cell kill occurred as suggested by the DI level (Chart 20).

The LI_{15} curves, following three ara-C injections, were somewhat different from what one may have predicted because the LI_{15} values of

the 5 hour groups were recovering at about the 5% level. One would have expected that the LI_{15} would remain inhibited for a longer time after the 3 ara-C injections than after a single administration (Chart 20). The LI_{15} curve regained the asynchronous control level by 13 hours, similar to that observed after a single ara-C injection (Chart 20). The MI, following three injections was explainable (Chart 21) inasmuch as the MI at 1 hour was in fact measured 3 hours after the first ara-C injection. Therefore, those cells that were in the latter segment of S and in G_2 at the time of drug administration had all passed through mitosis after the 3 hour interval had elapsed. The MI remained near the zero mark much longer after 3 than after a single injection of ara-C. It recovered rather dramatically by 17 hours after the last ara-C injection in both instances, and in subsequent groups had returned to the control level (Chart 21). Interestingly enough there was a shift in the peak DI to 5 hours when three doses of ara-C was administered, while that peak following a single ara-C injection occurred only after 17-21 hours. This can be interpretated to imply that three doses of ara-C were more effective in killing S phase cells; thus producing the DI peak at 5 hours, followed apparently by a second peak at 25 hours. Again this phenomenon is likely ascribable to the second mode of cell death reported by Whitmore et al, (1969). After a single injection of ara-C the DI peak occurred much later, for still unexplained reasons. It may imply that with these dosages fewer cells were actually killed in S, but temporarily inhibited, or perhaps the delayed DI peak was brought about by reasons not yet recognized. In both experimental series, the MI peak followed approximately 4 hours after the peak of LI_{15}. Theoretically, the peaks should have been at least 6 hours apart;

but since the intervals of data sampling was performed at 4 hour intervals. The actual peaks may have fallen somewhere between these sampling times.

A triple dosage of 60 mg/kg ara-C was considered to be the most effective chemotherapeutically (Chart 21), because the LI_{15} levels remained relatively low when compared to those of the other series; also, the degree of cell kill was the highest, as indicated by the DI values.

Both single and triple doses of 60 mg/kg ara-C, administered to a cell population previously synchronized and at a point when the largest proportion of cells were in the S phase following the release of ara-C in the synchronization procedure, effectuated an immediate decline of the LI_{15} curves (Chart 28). The curve produced by that single ara-C injection was shifted to the left by approximately 4 hours. The LI_{15} following immediately the single ara-C injection was somewhat lower than that observed in the original synchronized population. The 10 hour interval between the two LI_{15} peaks occurring after the ara-C treatment of the synchronized cell population may imply a shortening of the cell cycle from 14 to 10 hours. The single 60 mg/kg ara-C injection also reduced the MI curve by preventing cells destined to enter M from completing their DNA synthesis (Chart 29A). The small peak noted was likely ascribable to some escape of cells into M, however. The single ara-C dose produced little change of the DI from that of the untreated synchrony curves (Chart 29C).

The major effect of three doses of 60 mg/kg ara-C on the synchronized cell population is seen on the LI_{15} curve. The curve declined immediately by much fewer cells incorporating H^3-thymidine; further,

Chart 28.

LI_{15} curves of ara-C treated and untreated synchronized B16 melanoma. A. Synchronized population treated with a single 60 mg/kg injection of ara-C (Chart 22). B. Synchronized population treated with 3 single 60 mg/kg injections of ara-C (Chart 24). ⊙, asynchronous LI_{15} control level; ●, synchronized LI_{15} curve from series 4; O, treated synchronized LI_{15} curves;  , ara-C injections producing synchrony (series 4); △, marker groups for degree of synchrony and ↓, indicating ara-C injections at that point when most cells were in the S phase in the synchronized cell population.

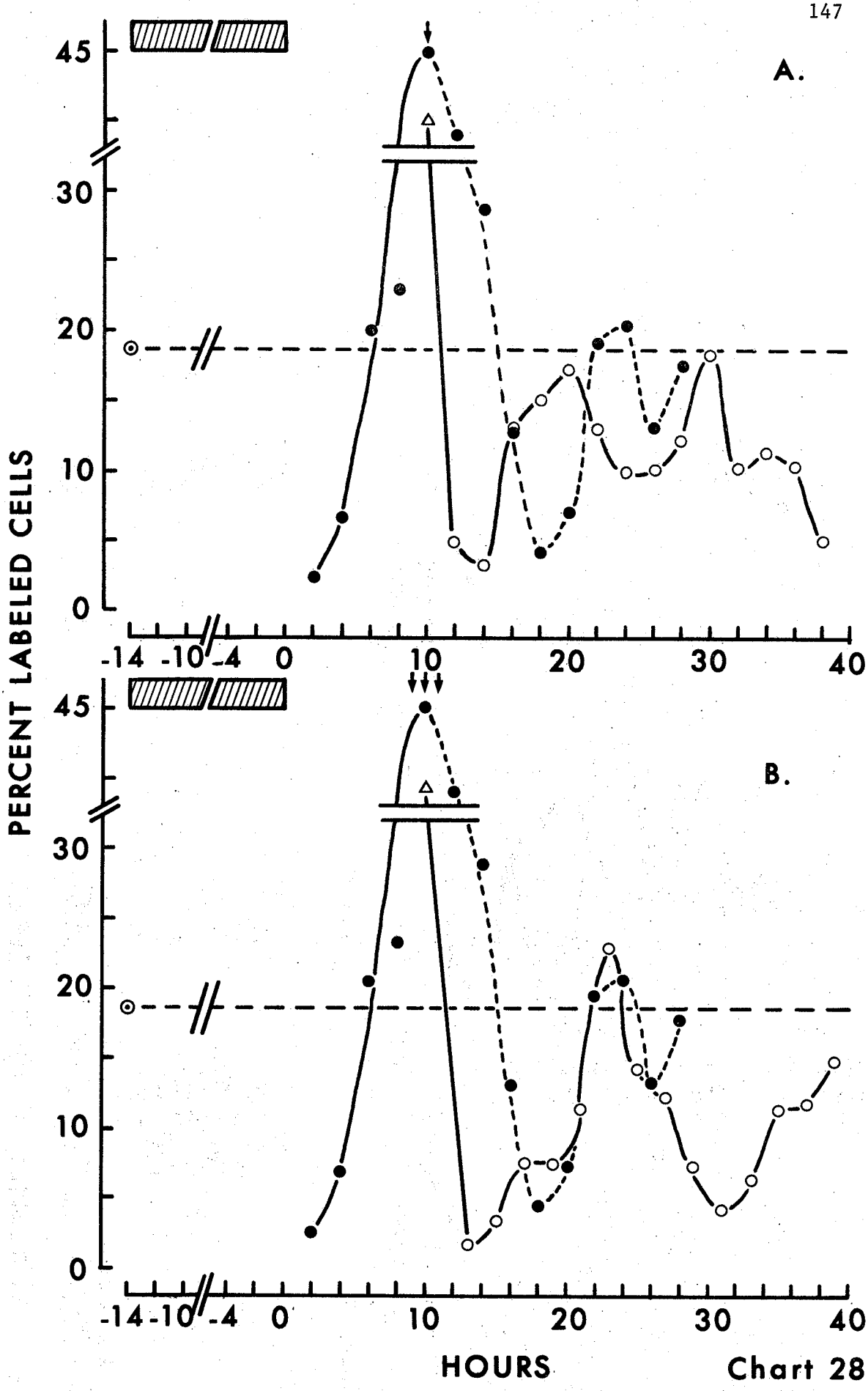


Chart 28

Chart 29.

MI and DI curves of ara-C treated and untreated synchronized B16 melanoma. A. MI curve of a synchronized population treated with a single 60 mg/kg injection of ara-C (Chart 22). B. MI curve of a synchronized population treated with 3 single 60 mg/kg injections of ara-C (Chart 24). C. DI curve of a synchronized population treated with a single 60 mg/kg injection of ara-C (Chart 23). D. DI curve of a synchronized population treated with 3 single 60 mg/kg injections of ara-C (Chart 23). ⊙, asynchronous MI and DI control levels; ●, synchronized MI and ■ DI curves from series 4; O, treated synchronized MI and □ DI curves; ▨, ara-C injections producing synchrony (series 4); △, marker groups for degree of synchrony and ↓, indicating ara-C injections at that point when most cells were in the S phase in the synchronized cell population.

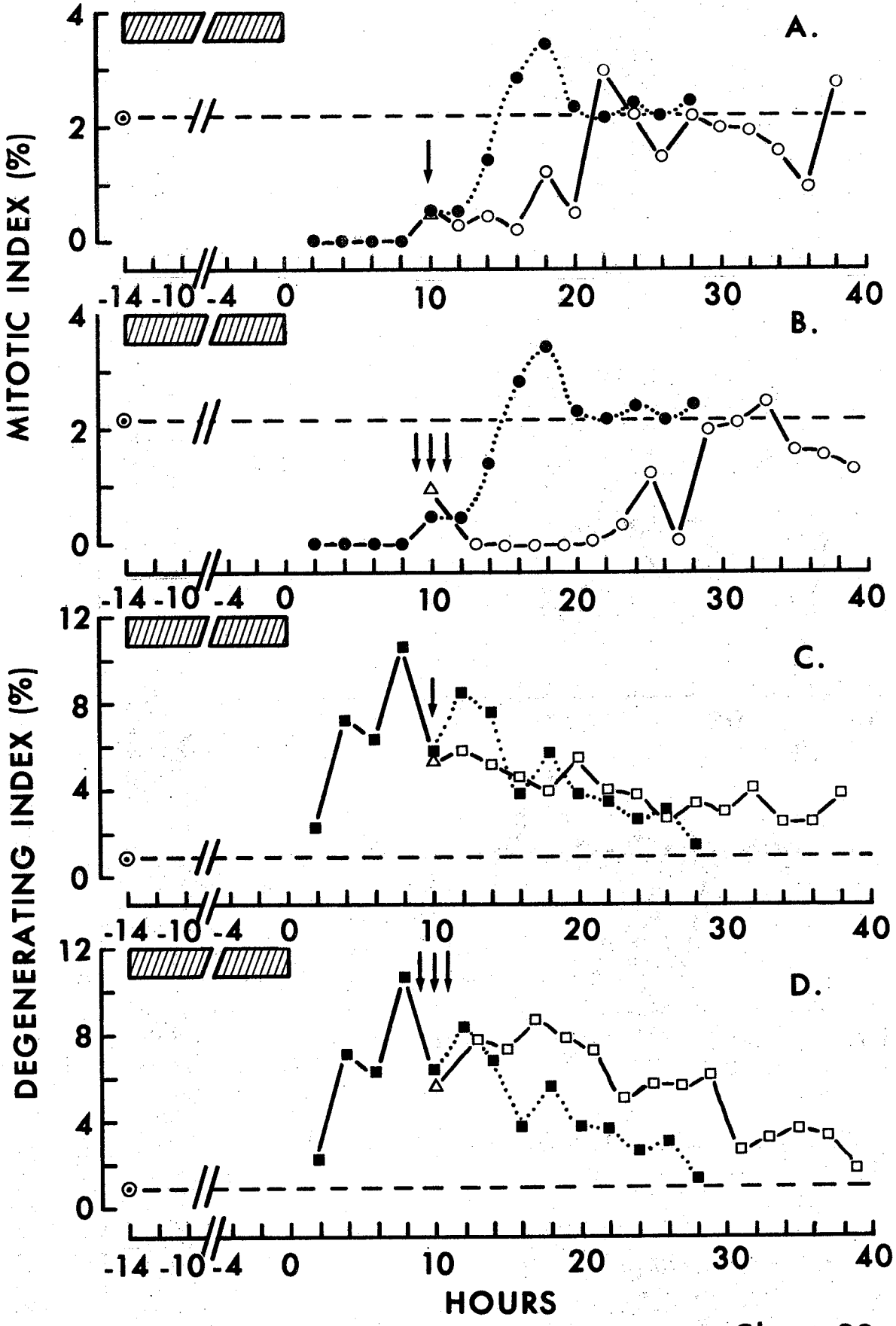

A.
B.
C.
D.
MITOTIC INDEX (%)
DEGENERATING INDEX (%)
0
2
4
8
12
-14 -10 -4 0 10 20 30 40
HOURS

Chart 29

there was a delay of 4-5 hours when compared to the untreated synchronized cell population LI_{15} curve. The former curve attained its peak at the same time as did the LI_{15} of the untreated synchronized population, and exceeded it in magnitude. The MI curve (Chart 29B) revealed that two different cell populations may have resulted from the 3 ara-C injections, the first attaining the maximum MI at 25 hours, the second at approximately 33 hours. Comparable peaks were noted also in the untreated synchronized population at 10 and 18 hours. The DI of the synchronized population, affected by the three ara-C doses, revealed an augmentation of the proportion of cells killed (Chart 29D).

All observations signify that three ara-C doses produced a higher degree of DNA synthesis inhibition and a more effective cell kill than a single dose in a synchronized cell population. Yet, all these effects were merely transitory. Perhaps the most significant information achieved by these last two experimental series was that cell synchrony, by the method described, was readily reproducible, inasmuch as the marker LI_{15}, MI and DI values (Charts 28 and 29) were statistically identical to those of series 4 ($p > 0.05$).

This study reveals that <u>in vivo</u> cell synchrony can be produced by inhibition of DNA synthesis of cells in the proliferating fraction of a malignant tumor by the cytostatic drug, cytosine arabinoside. The interpretations of the results were made based on the cell kinetics of asynchronous tumors, and therefore may have to be altered as more information is made available in the literature regarding the cell kinetics of synchronized tumor populations. It remains to be investigated whether the degree of synchrony can be improved upon or whether a tumor population with a larger growth fraction can be synchronized to a

greater extent. It also remains to be shown whether this method of cell synchrony will aid in the treatment of malignant growths.

CHAPTER VI

CONCLUSIONS

The present study was concerned with the normal cell kinetics and production of *in vivo* cell synchrony in B16 melanoma, employing the DNA synthesis inhibitor cytosine arabinoside. Further, the effects of ara-C upon synchronized B16 melanoma cell kinetics were examined. The principal observations were:

1. Growth of B16 melanoma was rapid during its early growth phases but declined as the tumor became older.

2. The decline of the growth rate of B16 melanoma was brought about partly by a prolonged generation time in the latter phases of growth, partly by a loss of tumor cells chiefly by central necrosis.

3. The growth fraction of B16 melanoma was 0.53; it remained stationary between days 7 and 16 of tumor growth.

4. Following a single administration of 50 mg/kg ara-C, the cell cycle duration of B16 melanoma on days 6 and 18 of tumor growth remained unchanged from that of the untreated tumors although the duration of the S phase of the treated tumor cells was shortened in both phases.

5. The highest degree of *in vivo* cell synchrony was achieved in the B16 melanoma on day 10 of tumor growth by eight injections of 12.5 mg/kg of ara-C at 2 hour intervals. The experimentally observed SI was 40.7, and the adjusted SI was 76.8.

6. *In vivo* cell synchrony of B16 melanoma was readily reproducible by administration of eight 12.5 mg/kg injections of ara-C at 2 hour intervals.

7. A direct relationship existed between the degree of the SI and the number of cells killed following a prolonged block of DNA synthesis by ara-C; cell kill was determined by the degree of the DI.

8. The decay of the *in vivo* cell synchrony occurred rapidly.

9. In order to achieve a high level of synchrony in the cell population, DNA synthesis should be blocked longer than 8 hours in B16 melanoma to allow all cells present in the latter part of S, in G_2 and M at the time of drug administration to pass through the cycle and accumulate at the junction between G_1 and S.

10. The cells killed by a prolonged DNA synthesis block were probably those that had passed through G_1 and had been accumulated for longer periods attempting to enter the S phase, thereby bringing about an "unbalanced growth" phenomenon.

11. Both single and triple injections of a larger dosage of 60 mg/kg ara-C effectuated the large DI and the lower LI curves in asynchronous B16 melanoma than smaller 40 and 50 mg/kg ara-C dosages.

12. Three 60 mg/kg ara-C dosages caused a higher degree of cell kill (DI) than an identical single administration in the previously synchronized B16 melanoma at a time when the highest percentage of tumor cells were in the ara-C sensitive S phase.

CHAPTER VII

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