**THE COMPUTER SIMULATION
OF LEUKEMIA THERAPY:
COMBINED PHARMACOKINETICS,
INTRACELLULAR ENZYME KINETICS,
AND CELL KINETICS OF THE TREATMENT
OF L1210 LEUKEMIA BY ARA-C**

PREPARED UNDER GRANTS FROM THE DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE

T. L. LINCOLN, P. F. MORRISON, J. AROESTY, G. M. CARTER

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PREFACE

Under Grants CA12369, CA14088, and CA19663 from the National Institutes of Health, The Rand Corporation is seeking to improve the biological precision of chemotherapy in the treatment of cancer, leukemia, and the lymphomas. The rationale is that mathematical models and computer simulations based on the biology of disease and its treatment can assist in the development of a clinically useful predictive approach to chemotherapy. They can also be used to organize, in a useful way, the burgeoning quantities of data arising from new assays, new diagnostic procedures, new technology, and new protocols.

Antitumor drugs are powerful, but they are not specific to malignant cells; they affect normal cells as well. Relatively small differences in chemical structure, dose, method of administration, and treatment intervals can lead to profound effects on the outcome of therapy. Fortunately, much can be learned from laboratory tests with experimental animals and cells grown in tissue culture. Experimental models are selected that share important characteristics with human clinical therapy. Indications of major side effects and toxicity, and of powerful anti-cancer and antileukemia activity, are the key experimental results and provide the scientific foundations for clinical progress. However, there are few direct biologically plausible rules for comparing these experimental results with clinical therapy, or for comparing or predicting the clinical responses of individual patients. The ties between basic science and clinical oncology are still tenuous ones, and the experimental model is the critical link.

As part of Rand's exploration of a decision-aiding methodology for use during the treatment of leukemia, the authors are investigating the feasibility of a computer-based leukemia therapy simulator. The present report grows out of that investigation, with application to experimental rather than to clinical therapeutics. As leukemia is the "window on cancer," so animal leukemia is the window on human leukemia.

Different animals, organs, and cell types have highly specific physical and biochemical properties that alter their response to therapy.

One aspect of these differences, the growth characteristics of cells both untreated and treated, is the theme of cell kinetics. Another aspect, the altered disposition and time history in critical body organs of the active forms of a drug, constitutes the subject of pharmacology and pharmacokinetics. A third aspect involves interactions among intracellular pharmacology and cell growth and kinetics, at the ultimate site of action. And a fourth aspect of this range of biological variability, less completely understood and much less quantifiable at present, involves the interplay among the immune system, the host, tumor growth, and the treatment plan. In this report, the mathematical models and computer simulations explicitly describe cell kinetics, pharmacokinetics, and intracellular pharmacology in a highly quantitative way; but the consideration of immune system interactions is necessarily more qualitative.

The authors describe a comprehensive computer model of the chemotherapy of leukemia, as applied to a specific drug, cytosine arabinoside (ara-C). This drug has already been instrumental in prolonging survival in adult leukemia, and suggestions are made here, on the basis of the simulations, for improving its clinical use.

The basic model applies to any animal species. However, the detailed measurements for incorporation in the simulations described here apply only to L1210 leukemia and mice. The simulations thus involve experimental therapeutics, while the discussion relates the results to the potential improvement of clinical therapy.

This report¹ should be of interest to specialists in quantitative biology and medicine: oncologists, hematologists, biophysicists, bioengineers, biomathematicians, pathologists, physiologists, pharmacologists, and medical students in the basic sciences. Other, related Rand publications include:

T. L. Lincoln, J. Aroesty, G. Meier, and J. F. Gross, "Computer Simulation in the Service of Chemotherapy," *Biomedicine*, Vol. 20, January 1974, pp. 9-16.

¹An abridged version of the report will be published in *Cancer Treatment Reports*.

J. Aroesty, T. Lincoln, N. Shapiro, and G. Boccia, "Tumor Growth and Chemotherapy: Mathematical Methods, Computer Simulations, and Experimental Foundations," *Mathematical Biosciences*, Vol. 17, 1973, pp. 243-300.

P. F. Morrison, T. L. Lincoln, and J. Aroesty, *The Disposition of ARA-C and Its Metabolites: A Pharmacokinetic Simulation*, The Rand Corporation, R-1757-NIH, August 1975.

J. Aroesty, T. L. Lincoln, W. S. King, and P. F. Morrison, *Hematological Compensation During Treatment of Sickle Cell Anemias: A Simple Mathematical Model*, The Rand Corporation, R-1952-HEW, November 1976.

J. Aroesty, T. L. Lincoln, P. F. Morrison, and Grace M. Carter, "Cell Kinetic Models of Transient States: A Preliminary Investigation of Lymphocyte Stimulation," to be published in *Proceedings, M. D. Anderson 29th Annual Symposium on Fundamental Cancer Research*, 1977.

SUMMARY

The chemotherapy of cancer and leukemia in laboratory test animals is known to be sensitive to details of the treatment plan. Both humans and laboratory test animals have been shown to exhibit such sensitivity, but only in the case of animal tumors have investigators been able to take advantage of this sensitivity to achieve extremely long-lasting remissions or cures. Such factors as drug composition and structure; drug dosage and schedule; the manner in which the drug is administered (i.e., oral versus intravenous, bolus injection versus long-term infusion, etc.); type, sensitivity, and resistance of malignant and normal cells; and the status of the immune system all contribute to the balance between undertreatment (dominated by the toxic effect of disease) and overtreatment (dominated by the toxic effect of treatment on normal tissue function).

Ideally, treatment decisions should, and in a few cases already have started to, reflect the importance of these factors for an individual patient. Presently, the effect of only some factors on treatment outcome is quantifiable. As a means of extending this quantification to the treatment of acute leukemia, we have been exploring the feasibility of developing a leukemia-therapy simulator. This report describes our progress in the development of such a computer-based simulator for the treatment of a commonly studied animal leukemia. The extension to the human clinical situation is much more complex, but the present study helps to point out those critical factors for individual patients which must be measured and quantified before a realistic attempt at a comprehensive predictive simulation of the therapy of human leukemia can be attempted. These factors bear particularly on specific modes of sensitivity and resistance of leukemic and normal cells to treatment, and the impact of immune response on cell proliferation under therapy.

Our approach is a modular one: individual computer-based mathematical models of cell growth, drug distribution, intracellular biochemistry, and cellular lethality are developed and individually tested

based on relevant biological measurement and similitude. Once the component models have been verified, they are then integrated into a comprehensive model of treatment. In this way, measurements and data obtained from *in vitro*, *in vivo*, and even clinical tests have been organized to define quantitative interactions and to enhance predictability.

This report describes an integrated mathematical computer-based model of the pharmacokinetics, intracellular enzyme kinetics, and cell kinetics of the treatment of L-1210 mouse leukemia by cytosine arabinoside (ara-C). The compartment model of Bischoff, Dedrick et al. (1971) is extended to the intracellular level by inclusion of equations describing the phosphorylation of ara-C, controlled by deoxycytidine kinase; the deamination of ara-C to its inactive form ara-U, controlled by deaminase; and the dephosphorylation of ara-C mono-, di, and triphosphate, controlled by phosphatase. The activities of kinase, deaminase, and phosphatase are explicitly included in the models and are estimated from relevant data. These activities are a quantitative aspect of drug resistance, and corresponding values in the clinical case are required for further progress in extending this approach to human treatment.

Cell proliferation is described by a continuous flow mathematical model in which cellular maturation and cell-to-cell variability in maturation rates are key variables. Cell proliferation is related to intracellular biochemistry through mathematical expressions which relate cell lethality and progression delay to the time course of intracellular ara-CTP. These expressions are consistent with both unbalanced growth and incorporation modes of cell death. Simulations of *in vitro* and *in vivo* experiments performed in a number of laboratories are described.

The most sensitive parameters in dose response and cell survival simulations are deoxycytidine kinase activity, ara-CTP half-life, renal clearance of ara-C, and cell-kinetic parameters describing cell proliferation and cell killing. Progression delay is vital to the realistic simulation of divided-dose schedules. All of these factors which play important roles in potential variability of therapeutic effectiveness must be estimated prior to extending this methodology to humans.

Further implications of combined simulation for relating pharmacokinetic, biochemical, and cell-kinetic *in vitro* and *in vivo* data, for exploring consistency among different measurements, and for relating animal experiments to clinical treatment are also described in this report.

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I. INTRODUCTION

The impact of chemotherapeutic agents on neoplastic tissues can be most easily studied using animal models and tissue culture techniques. However, this experimental information must be integrated in a self-consistent way if it is to be used in the rational treatment of human tumors.

We are attempting, by means of simulation, to develop an idealized picture of the physiological, biochemical, and proliferative events that mark the treatment of a growing tumor by a cycle-sensitive agent. Our objective is to build a simulated model that will scale to other experimental tumors and that can ultimately describe chemotherapy in man. To this end we have formulated our mathematical models in such a way that all of the important intermediates are documented in quantitative detail and are individually calibrated by comparison to measurement.

The prototype biological system is the impact of cytosine arabinoside (ara-C) on the L1210 mouse leukemia, chosen because of the richness of data available. Our approach compels us to consider the links between the biochemical mechanism of action of ara-C and the cell kinetics of the proliferative cycle. Thus our simulations rely on more mathematical structure and contain more detail than the classic models of cell kinetics. By means of this structure, cell-kinetic and biochemical kinetic models of susceptibility and resistance can be quantified and directly compared with laboratory data.

II. BACKGROUND

Cell-kinetic models were first introduced to describe the kinetic relationship between DNA synthesis and mitosis. Experimental protocols used radiolabeled DNA precursors to label DNA synthesis and to trace the passage of cells through mitosis. The limited number of observables dictated the simple structure of these models. Cells could be assumed to pass through a short sequence of proliferational states. Either a cell became labeled, or it did not. Either it was in mitosis, or it was not. Either it divided, or it did not. Stochastic rules provided the flux over time of this sequence of events. Later, when more subtle concepts were introduced to account for new observations involving growth fraction, cell loss, and cell death, they were added to the original stochastic models in an ad hoc way. Models that were adequate to interpret the proportion of labeled mitosis following a short exposure to radioactive nucleotides became more complex when it became evident that cells of the same origin underwent DNA synthesis and division at different rates. Computer-based programs superseded the original simple methods of interpretation.

These computer-based models have become more or less standardized. Because of this standardization and their widespread availability, they are used in the interpretation of labeled mitosis measurements. However, the underlying kinetic structure is no more refined than the original intuitive models of two decades ago. Stochastic compartment models do not possess enough biological detail for use in studies involving chemotherapy, DNA content, activation, or the apparent variability of thymidine incorporation with nuclear maturity. New biological problems require new solutions. In order to describe cycle-sensitive chemotherapy, we require models that meld biochemistry and cell kinetics.

The paradigm of the cycle-sensitive agent is cytosine arabinoside, which is widely used in experimental models and is effective in the treatment of human leukemia. Its pharmacokinetics, intracellular pharmacology, and biochemistry have been characterized both by laboratory and clinical measurements. In an earlier report (Morrison et al.,

1975), the pharmacokinetic distribution of ara-C into tissues and the biochemical kinetics of phosphorylation, dephosphorylation, and deamination were simulated in detail. The present report continues our earlier work by simulating the effect of ara-C and its metabolites on the proliferative events in a growing tumor. Figure 1 summarizes the relation of this work to our earlier pharmacokinetic study.

Detailed models of drug distribution, excretion, and metabolism are used to show the pharmacokinetic disposition of ara-C and its intracellular metabolites, the mono-, di-, and triphosphates. The conceptual basis for these simulations is the pioneering work of Bischoff et al. (1971), which we have extended to the intracellular level using the mixed inhibition enzyme kinetics of Dixon and Webb (1964). Our initial calibration studies simulated the extensive tissue-specific data of Chou et al. (1975), obtained for the DBA mouse including tissue and blood levels of ara-C, the active form of ara-CTP, and the metabolite ara-U. Chou et al. provided no renal clearance data, and the value suggested by Dedrick et al. (1973) proved to be too low. A higher value, well within the physiological range, was consistent with both Chou's work and the ara-C blood levels measured by Borsa et al. (1969). Figure 2 summarizes these earlier results.

The most significant finding by experiment and by simulation is the ara-CTP level in tissue, which decays much more slowly than the ara-C levels in blood. For example, at a dose of 5 mg/mouse, ara-CTP levels in the spleen are at cytotoxic levels for 11 hours and can appreciably inhibit the synthesis of DNA for more than 15 hours. This is contrasted with negligible blood levels of ara-C after 5 hours. Because of the rapid phosphorylation of ara-C and the long half-life of ara-CTP, ara-CTP levels are closely related to peak ara-C levels in tissues.

The growing tumor that we simulate is the L1210, chosen primarily because (1) its enzyme levels and nucleotide ratios have already been measured; (2) the data of Bhuyan et al. (1973), Edelstein (1975), Edelstein et al. (1974), and ultimately Skipper et al. (1970) provide a matrix of experimental data for both calibrating and testing our simulation; and (3) our basic cell-kinetic approach to simulation had

Fig. 1— Simultaneous simulation of pharmacokinetics, intracellular enzyme kinetics, and cell proliferation kinetics using mathematical computer-based descriptions of drug distribution; excretion and metabolism; the net conversion of ara-C to its active form within the cell, ara-CTP; and the effect on tumor cell survival of the time history of ara-CTP

Fig. 2—Pharmacokinetic simulation of ara-C and splenic ara-CTP. (Intracellular levels of ara-CTP that are either cytotoxic or cytostatic persist well after blood levels of ara-C have decayed to insignificance.)

already been verified by our investigation of Hartmann's Puzzle for this tumor (see Sec. V).

Other experimental tumors with smaller growth fractions would, of course, be more consistent with the current view of clinical disease for primary tumors, but the availability of needed biochemical data, and the possibility of *in vitro-in vivo* correlation for both progression delay and cell lethality, made L1210 a natural starting point.

The pharmacokinetic models draw attention to factors that are sometimes overlooked in animal-model experimental protocols. For example, the convenient injection of .5 cc of a drug into a 20-gram mouse is equivalent to 1.75 liters by very rapid infusion in man. The consequent augmentation of renal clearance will lower the blood level of ara-C. More importantly, the use of intraperitoneal injection to treat ascites tumors can have a significant therapeutic advantage because it exaggerates the exposure to active drugs many hours beyond the transient difference in ascites/blood concentration¹ (Chou et al., 1975). This goes a long way toward explaining the enormous success of Skipper's (1970) ara-C protocol on L1210 ascites cells at dose levels that are not as effective if the L1210 is grown in the femur or if the same dose is given by some other route. This therapeutic advantage cannot be extrapolated to man.

For this study, the previously calibrated pharmacokinetic model is used to calculate intracellular levels of ara-CTP. A central assumption is that both lethality to S-phase cells and progression delay (the reduction in DNA synthesis rate) are related to the time history of intracellular ara-CTP concentration.

As the drug is incorporated into proliferating cells, the ara-CTP concentration has its measured effects on the cell population. The behavior of a surviving fraction is then simulated as a function of ara-CTP history and cellular maturation.

¹Our simulations show that the net conversion of ara-C to ara-CTP is sufficiently fast that the high peritoneal concentrations of ara-C for short times after intraperitoneal injection lead to increased "c x t" for ascites cells.

III. METHODS OF PROCEDURE

Our experimental approach is one of mathematical simulation. We use and construct mathematical models which have parameters that can be specifically related to experimental data. These are united into simultaneous computer simulations of pharmacokinetics, intracellular biochemical kinetics, and cell kinetics (see Fig. 1).

The approach to pharmacokinetics is designed to provide the concentration of an active drug at its site of action as a function of time. To model *in vivo* the tissue-by-tissue distribution, metabolism, and excretion of ara-C, we have modified the Bischoff-Dedrick physiological multicompartment model for the flow and limited exchange of ara-C between organs and blood supply.

The simulation of intracellular enzyme kinetics applies to both *in vivo* and *in vitro* conditions. The minimal set of pharmacokinetic and enzymatic relations for describing the intracellular concentrations of ara-CTP are included. Equations are programmed which describe the phosphorylation of ara-C, controlled by deoxycytidine kinase; the deamination of ara-C to an inactive form, ara-U, controlled by nucleoside (cytidine) deaminase; and the dephosphorylation of ara-C mono-, di-, and triphosphate controlled by nucleotidase. The enzyme levels of the kinase, nucleotidase, and deaminase are included explicitly in the simulations, and ultimately determine the level and time course of ara-CTP. In this way, enzymatic modes of resistance and variability are quantified, and the sensitivity of ara-CTP to enzyme variation is studied. The details of this approach have already been described (Morrison et al., 1975). A typical equation, shown here for convenience, represents the enzymatic conversion of ara-C, feedback pathways, and inhibition by invoking the mixed inhibition formalism of Dixon and Webb (1964);

$$\frac{d}{dt} (ap_3) = \left(\frac{1}{\alpha} + 1 + \frac{1}{\alpha_2 \alpha_1} \right)^{-1} \left(\dot{r}_p + \dot{r}_{dp} \right) \quad (1)$$

where the phosphorylation rate is given by

$$\dot{r}_p = \frac{V_k}{1 + \frac{K_m}{a} \left(1 + \frac{c}{K_i} + \frac{dCR}{K_I} + \frac{ap_3}{K'_i} \right) + K_m \left(\frac{c}{K_i K'_m} + \frac{ap_3}{K'_i K''_m} \right)} \quad (2)$$

and the dephosphorylation rate is given by

$$\dot{r}_{dp} = \frac{-V_{dp}}{1 + \frac{\alpha_1 \alpha_2 K_{dp}}{ap_3}} \quad (3)$$

where the notation is described in our earlier report (Morrison et al., 1975).

The approach to cell kinetics is designed to interpret modern kinetic measurements of DNA content which divide a cell population according to maturity. It is also designed to deal with variations in rates of DNA synthesis or labeled nucleotide incorporation as in progression delay or lymphocyte activation.

We assume maturation to be a continuous process and individual cells to differ in their rate of maturation. In order to interpret the perturbation of cell-kinetic systems, the distribution of cells over cycle times and the distribution of cells according to the proliferative maturity must be explicitly represented and followed as a function of time.

Rubinow's maturity-time representation (1968) has been extended (Aroesty et al., 1973) as a continuous flow maturation model to represent cell proliferation. Intercell variability in maturation rate is explicitly included in this description.

The fundamental variable is the cell density function $n(t, \mu, T)$ where t is time, μ is maturity, and $1/T$ is the specific rate of maturation. Rather than deal with maturation rate, it is convenient to deal with its inverse, T . If the rate is independent of maturity,

then T corresponds to cell-cycle traverse time. The total number of dividing cells is

$$N(t) = \int_{T_{\min}}^{T_{\max}} \int_0^1 n(t, \mu, T) d\mu dT, \quad (4)$$

where $T_{\min}$ and $T_{\max}$ correspond to the minimum and maximum cell-cycle times.

The maturity variable μ relates to continuous landmarks in the cell cycle, i.e., $\mu = 0$ corresponds to newly divided cells, and $\mu = 1$ corresponds to initiation of mitosis. DNA content or cell volume might be appropriate measures of μ in different parts of the cycle.

The rate of cell maturation is related to the elapsed time since cell birth or age, a , according to the equation

$$d\mu = \frac{da}{\left[\frac{T}{\alpha(\mu, t)} \right]} \quad (5)$$

where $\alpha(\mu, t)/T$ is the rate of maturation in the presence of drug effects. If no drug effect is present, then $d\mu = da/T$.

The basic cellular conservation law for describing cell proliferation is essentially

$$\left[\begin{array}{c} \text{the change in the} \\ \text{number of cells} \\ \text{of a given} \\ \text{maturity} \end{array} \right] = \left[\begin{array}{c} \text{the difference between} \\ \text{the number of cells} \\ \text{that, as they} \\ \text{age, mature into and} \\ \text{out of this maturity} \\ \text{level} \end{array} \right] - \left[\begin{array}{c} \text{the number of cells} \\ \text{of this maturity} \\ \text{level that are} \\ \text{killed} \end{array} \right]$$

While the continuous maturity variable, μ , and the maturation rate, $\alpha(t,\mu)/T$, are appropriate conceptual variables for describing this model, it is sometimes more convenient to replace μ by its equivalent in age, a , using Eq. (5).

The fundamental equation for the cell-density function $n(t,\mu,T)$ becomes

$$\frac{\partial n}{\partial t}(t,\mu,T) + \frac{\partial}{\partial \mu} \left[\frac{\alpha(\mu,t)n(t,\mu,T)}{T} \right] = -\lambda(t,\mu,T)n(t,\mu,T) \quad (6)$$

where λ is the cell-death function and represents both drug effects and natural tumor attrition. This must be supplemented by a condition describing cell birth. In general, we write:

$$\frac{n(t,0,T)}{T} = \beta \int K(T,T') \frac{n(t,l,T')}{T'} dT' \quad (7)$$

where β is the average number of daughter cells per division and $K(T,T')$ is a kernel function which relates the maturation rate of a daughter cell to that of its parent. Evidence to determine $K(T,T')$ is scant, but there is important experimental evidence (Valleron, 1975; Prescott, 1959) to show that heredity cannot be ignored. Experimental and clinical evidence also suggests a selection of cells by cycle time as a result of chemotherapy (Omine et al., 1973). Labeled mitoses data are generally insensitive to the degree of correlation between the cycle times of successive generations unless many cycles can be followed. The implications of heredity and its connection to Rubinow's Paradox are described in Sec. V.

In the present simulations, correlations between parents and offspring have been neglected. The simplest version of the kernel function, consistent with observations on the distribution of maturation rates, is

$$\frac{n(t,0,T)}{T} = \beta G(T) \int_{T_{\min}}^{T_{\max}} n(t,l,T') \frac{dT'}{T'} . \quad (8)$$

$G(T)$ was chosen to be a displaced gamma distribution, described by a mean cycle time $\bar{T}$, a minimum cycle time $T_{\min}$, and a coefficient of variation.

It is helpful, for purposes of visualization, to replace maturity, μ , by its equivalent in terms of age, a , or elapsed time from last mitosis. This is done using the relationship from Eq. (5), in the absence of drug effect,

$$\frac{da}{d\mu} = T . \quad (9)$$

The domain of the cell-density function at a particular time, t , is illustrated in Fig. 3. The volume bounded by the cell-density function corresponds to the total number of cells. The phases of the cell cycle, G_1 , S , G_2 , and M are shown as wedge-like regions. This is a simplification and is not vital to our approach. However, our investigation of labeled mitosis for Ll210 and JBl, based on data presented by Dombernowsky and Hartmann (1972), demonstrates that a satisfactory simulation could be obtained if the duration of each phase of the cell cycle was approximated as a fixed fraction of the cycle time.¹ This is also compatible with the experiments of Miller and Osmond (1973) on lymphocyte proliferation in the guinea-pig bone marrow, where the duration of S-phase increases with the cycle time. The location of the cell-cycle phases in maturity space thus appears to be independent of cycle time. A consequence, of course, is that the durations of the individual phases of the cell cycle are highly correlated.

¹For other systems, which are not so proliferative and where G_1 rather than S is the major determinant of cycle time, our simulations can reflect the known greater variability in G_1 rates.

Fig. 3—Domain of the cell-density function, a three-dimensional representation of the dependence of the cell-density function on cell age and cycle time. The total number of cells is related to the volume covered by the curved surface. When the age, a , equals the cycle time, T , then cells divide resulting in β daughter cells per division

As cell populations become more dense, there are changes in cell-cycle time distributions and in the proportion of cells that do not cycle or that die. These phenomena are among those most indirectly measured and incompletely understood. In the present work, we consider only rapidly growing cell populations which may exhibit cycle prolongation and increased cell death but for which it appears unnecessary to make added assumptions about a G_0 phase to account for a change in growth fraction.

Our models consider natural attrition, the accumulation of nonviable cells, and changes in the attrition rate due to ara-CTP. Thus, we are prepared to analyze differences in cell loss in early, rapidly growing tumors and in older ascites tumors where the cells are denser and the behavior exhibits relatively slow net growth.

The general formulation is based on analysis of Dombernowsky's extensive experimental data of both the L1210 and the JB1 tumors for different phases of growth. We will briefly return to this problem in Sec. V in a form of model calibration which we term Hartmann's Puzzle.

For those systems examined to date, natural attrition of tumor

cells can be described by assuming that there is a subgroup of cells that cannot divide successfully but that can mature to the point of division without further DNA synthesis. These are sterile cells. Such cells may be generated by some intrinsic failure rate in the course of proliferation. It may be hypothesized that some form of host "hostility," perhaps the result of immune mechanisms, is responsible for this sterilization rate. In any case, cells no longer able to divide appear to accumulate as end-stage cells before they die.

For the present studies of the L1210 during exponential growth, almost all cells appear to be in cycle and the rate of natural attrition is no greater than 1 percent per hour. For the tumors we have studied in detail, the rate of this natural attrition appears to be independent of cycle time.

Significant attrition due to host hostility might well be enhanced if cycle times were prolonged. This may in part account for the increase in end-stage cells observed in tumors that are in a highly damped growth phase. Drugs that cause progression delay (by decreasing DNA synthesis) should also enhance this effect. Our model formulation allows us to look for these interactions and to model them.

In addition to the natural attrition or sterilization rate, it is necessary to develop a quantitative relationship between cell death and the intracellular history of ara-CTP concentration. This involves a cell death function, $\lambda(t, \mu, T)$, which should be related to ara-CTP concentration over time and to proliferative kinetics. Measurements have not yet provided a precise guide for this relationship. However, some significant data are available. The form of ara-C responsible for killing cells is ara-C triphosphate, but the exact means by which this compound exerts its lethal effect is not known. One suggestion (Karon et al., 1972) is that ara-CTP is incorporated into DNA to produce lethal events. In the presence of ara-C, DNA synthesis is largely blocked by the inhibition of DNA polymerase by ara CTP. Nevertheless, some ara-CTP residues are incorporated into newly formed sections of DNA (Silagi, 1965; Graham and Whitmore, 1970). This would eventually result either in the inability of a cell to produce correctly sequenced mRNA, or in chain termination. Presumably, the cell could not long cope

with either of these occurrences, which would then constitute lethal events. Another suggested mechanism is unbalanced growth: the inhibition of DNA synthesis by ara-CTP while protein and RNA synthesis continue generally unaffected may be the lethal event. However, the exact mechanisms by which this leads to structural or metabolic breakdown remain unelucidated.

It is consistent with both incorporation and unbalanced growth to postulate that the cell-kill function depends on the cumulative effect of ara-CTP acting on the cellular mechanisms that are responsible for loss of viability. The simplest form of cumulative effect is an integral over time. If the cells do not progress through cycle, this suggests that an appropriate form is

$$\frac{d\lambda}{dt} = \frac{F(\mu)(ap_3)^{k_1}}{K_2 + (ap_3)^{k_1}} - k_3\lambda. \quad (10)$$

However, since some cells do in fact progress through cycle in the presence of ara-CTP, the appropriate generalization is

$$\frac{\partial \lambda}{\partial t} + \frac{\alpha(t, \mu)}{T} \frac{\partial \lambda}{\partial \mu} = \frac{F(\mu)(ap_3)^{k_1}}{K_2 + (ap_3)^{k_1}} - k_3\lambda. \quad (11)$$

This extension of Eq. (10) reflects the requirement that λ be an intrinsic cell property, dependent on intracellular events. $F(\mu)$ is a maturity-dependent sensitivity function, and k_1 , K_2 , and k_3 are constant parameters.

The first term on the right-hand side of Eq. (11) is positive and always leads to an increase in the cell-death function. Cells can be exposed to high levels of drugs for a short time without toxicity; thus the second term, $k_3\lambda$, which always decreases the cell-kill function,

allows cells to recover from low-level damage caused by ara-CTP. If ara-CTP is removed, the cell-kill function approaches zero with a half-life which varies inversely with k_3 .

The sensitivity factor $F(\mu)$ has been introduced to account for the S-phase specificity of ara-CTP. Kim et al. (1968), for example, have shown that HeLa cells treated with ara-C in mid-S-phase are at greater risk than cells at the beginning or end of S-phase. $F(\mu)$ is nonzero only during S-phase, thus allowing $\lambda(\mu, t)$ to increase in value only during this phase. It is assumed that $F(\mu)$ rises from zero at the beginning of DNA synthesis, is nearly constant over most of S-phase, and then decays symmetrically to zero at the end of the synthetic period.

If the maturity at the beginning of S-phase is defined as μ_{G_1} and at the end of S-phase as μ_S , then $\mu_S - \mu_{G_1}$ is the maturity interval corresponding to DNA synthesis. The sensitivity function is represented by

$$F(\mu) = k_5 \left\{ \exp \left[-\frac{1}{2} \frac{(\mu - \mu_{G_1} - k_4)^2}{k_4^2} \right] - \exp \left(-\frac{1}{2} \right) \right\} \quad (12)$$

in region A, defined by $\mu_{G_1} \leq \mu \leq \mu_{G_1} + k_4$;

$$F(\mu) = k_5 \left[1 - \exp \left(-\frac{1}{2} \right) \right] \quad (13)$$

in region B, defined by $\mu_{G_1} + k_4 \leq \mu \leq \mu_S - k_4$; and

$$F(\mu) = k_5 \left\{ \exp \left[-\frac{1}{2} \frac{(\mu - \mu_S + k_4)^2}{k_4^2} \right] - \exp \left(-\frac{1}{2} \right) \right\} \quad (14)$$

in region C, defined by $\mu_S - k_4 \leq \mu \leq \mu_S$. The shape of the sensitivity function is exhibited in Fig. 4.

This general shape is required to allow cells blocked in early S-phase due to the inhibition of DNA polymerase to remain there at a reduced risk of death relative to mid-S-phase cells. This in turn accounts for the observation of Kim et al. (1968) that cells may accumulate in early S-phase for several hours following low-dose exposure to ara-C without showing significant death. The present model (Eqs. (6) and (11)) eventually permits these blocked cells to be killed, but only after they have progressed into the more sensitive part of S-phase.

The equation for $\lambda(\mu, t)$ is most easily interpreted in the case where the primary lethal event is the incorporation of ara-CTP into DNA. $\lambda(\mu, t)$ would then be assumed proportional to the amount of ara-CTP incorporated into potentially lethal sections of DNA at any instant of time, and $F(\mu)$ would be identified with the maturity-dependent probability of incorporating an ara-CTP molecule into such a lethal gene. Although the probability of death will not be proportional to incorporated ara-CTP over all levels of incorporation, the assumption of proportionality can be a good approximation to the initial part of a more reasonable probabilistic relationship between death and incorporated ara-CTP, such as a log-normal distribution. The term $ap_3^{k_1}/(K_2 + ap_3^{k_1})$ would represent the kinetics of incorporation generalized to include the case of allosterism, and $k_3\lambda$ would represent the effects of excisional repair. The parameter k_3 would not depend on ara-CTP because it has been shown (Rama Reddy et al., 1971) that the repair enzyme, at least in *E. coli*, is not inhibited by ara-CTP.

Such explicit interpretation of terms is not possible for the unbalanced growth mechanism. Here Eqs. (6) and (11) can only be viewed as a generalized "c x t" formalism.

It is stressed that these equations represent a plausible and/or empirical description of cellular loss due to the action of ara-CTP, since for either mechanism of death, insufficient quantitative detail about lethal mechanisms is known. The key underlying assumption in this model is that death is determined uniquely by the concentration history of ara-CTP and that the significant differences between cells

in their ability to respond to ara-CTP rest on their varying abilities to synthesize or degrade ara-CTP. If this assumption is valid, only these differences must be taken into account to provide a plausible and realistic simulation of cell death.

Fig. 4—Shape of the ara-CTP sensitivity function $F(\mu)$, in maturity space. $F(\mu)$ is zero in G_1 , G_2 , and M , gradually rises to its maximum in S , and then decays symmetrically

Finally, the means by which ara-C-induced progression delay is represented is highly simplified. The degree of inhibition of DNA synthesis of L1210 (and other cells) relative to untreated controls has been determined experimentally by Chou et al. (1975) as a function of ara-CTP concentration. The rate of maturation in S-phase has therefore been assumed to be reduced from the normal value $(1/T)$ by an amount proportional to the percent inhibition determined by Chou at a given ara-CTP concentration. Thus, $\alpha(\mu, t)$ is determined by the line drawn through Chou's data shown in Fig. 5 for S-phase and by unity outside of S-phase.

ESTIMATION OF PARAMETERS

The most difficult area is the estimation of constants and parameters for the various model components. This is an exercise in

Fig. 5—Relationship between progression delay and ara-CTP levels in mice, using Chou's (1975) data. The fit to Chou's data, which was based on thymidine incorporation, is used to determine $a(\mu, t)$ in Eq. (6)

consistency and plausibility, requiring careful comparison of conflicting and often incomplete data obtained from different laboratories under differing experimental conditions.

To model the effect of ara-C on the L1210, we examined the *in vitro* experiments of Bhuyan et al. (1973), the survival curves for L-cells of Graham and Whitmore (1970), and the L1210 *in vivo* measurements by Chou et al. (1975), Edelstein (1975), and Edelstein et al. (1974).

Enzyme activities appear explicitly in the equations governing the time history of ara-CTP, but the requisite activity data has not yet been gathered for these particular L1210 strains which are used in survival and progression delay studies. A sensitivity analysis of these equations (Morrison et al., 1975) indicated that deaminase activity could be estimated from ara-C/ara-U ratios in plasma; kinase activity could be estimated from short-term (one hour) measurements of ara-CTP and the insensitivity of predicted values to phosphatase levels; and phosphatase activities estimated from the measured ara-CTP half-life at longer times (eight hours). It was found that consistency across different laboratories and different L1210 strains could be established

by permitting kinase activity to assume different numerical values for each laboratory.

This must be considered a provisional resolution of the problem of inadequate enzyme activity data. Until such data are available, the resolution is supported by the observation that salvage enzymes such as deoxycytidine kinase are suppressed in different cell lines. The original extract values of Ho (1973) had been shown earlier (Morrison, 1975) to lead to accurate simulations of ara-CTP levels in normal murine tissues, but it was necessary to increase the Ho-Schrecker value for L1210 cells ($V_k = 16.6 \mu\text{M}/\text{min}$) by a factor of four to accurately simulate Chou's (1975) measurements on ascites L1210 cells.

Chou's measured relationship between DNA synthesis rate and ara-CTP levels is the quantitative basis for the representation of progression delay (Fig. 5). Bhuyan's (1973) measurements of cellular survival and dose response *in vitro* form the quantitative basis for the representation of the cell kill function (Eq. 5). The expression is in terms of ara-CTP concentration, a quantity not measured by Bhuyan. Bhuyan did however measure DNA synthesis inhibition as a function of ara-C levels at one hour. If ara-CTP is responsible for inhibition of DNA synthesis, then the correspondence between Chou's and Bhuyan's measurements, combined with an enzyme kinetic simulation of Bhuyan's experience, could be used to establish kinase activity for Bhuyan's culture. The kinase activity ($V_k = 2.67 \mu\text{M}/\text{min}$) required to maintain correspondence between Chou's and Bhuyan's measurements was far less than our estimate of Chou's value, or even the extract value measured by Ho and Schrecker.

Measurements by Edelstein (1975)¹ represent a physiologically relevant *in vivo* model for the treatment of L1210. Tumor cells growing in the femoral cavity of the mouse are treated with ara-C, and then transplanted to assess viability by means of the spleen colony assay. Simulations of these experiments were performed by using numerical values for organ sizes, blood flows, clearance rate, and enzyme activities of normal murine tissue taken for our earlier study (Morrison, 1975). Consistency between Edelstein's survival data at one mg/mouse

¹ See also Edelstein et al. (1974).

and the *in vitro* data of Bhuyan was then established by simulation, using a kinase value of $V_k = 16.67 \mu\text{M}/\text{min}$, intermediate to the Chou and Bhuyan values.

The intracellular half-life of ara-CTP determines total length of exposure to cytotoxic effects and progression delay. We employed a half-life of about two hours, a value consistent with our earlier pharmacokinetic simulations. While this is twice the ara-CTP half-life measured in HeLa cells (Momparker et al., 1971), this larger value coupled with the kinase value of $67 \mu\text{M}/\text{min}$ for ascites cells, and the Ho extract value for liver tissue, simulated Chou's measurements on both normal and tumor cells. The combination of greater kinase activity and shorter ara-CTP half-life (phosphatase) could perhaps account for the percent inhibition of DNA synthesis remaining at eight hours; such parameters would over-predict ara-CTP tissue levels after one hour of exposure.

Cell cycle coefficients for the L1210 were obtained from simulation and comparison with data of Dombernowsky and Hartmann. For both L1210 and JBI, we found (and will present in future reports) that a distribution of cycle times corresponding to a displaced gamma distribution characterized by three parameters--minimum cycle time, mean cycle time, and coefficient of variation--was appropriate for simulation. The coefficient of variation, the fraction of cycle time spent in each phase of the cell cycle, the difference between the mean time of transit and the minimum cycle time, and the sterilization rate (to be discussed below) were all independent of tumor size. The mean cycle time increased with increasing tumor burden in a way that was consistent with damped (Gompertzian) growth. The maturity intervals corresponding to the different phases of cycle could be approximated by constants, μ_{G_1} , μ_S , μ_M , independent of individual cell cycle time. Thus, the phase durations are highly correlated.

The average cycle time of L1210 cells (five-day tumor) of 12 hours and a coefficient of variation of the cycle time distribution of 0.3 were obtained from simulation and comparison with data of Dombernowsky and Hartmann. The S-phase interval in maturity space is defined by $\mu_{G_1} = 0.2$ and $\mu_S = 0.92$.

IV. RESULTS

Our results consist of detailed comparisons of simulations to data. These comparisons impose a hypothetical consistency on the data from many laboratories and focus on the parameters that appear inconsistent. Of necessity, these are the parameters to which the simulations are sensitive.

The most sensitive parameters are:

- o The deoxycytidine kinase activity
- o The ara-CTP half-life
- o The renal clearance of ara-C
- o The shape of the ara-CTP kill function
- o The distribution of cells in the cell cycle perturbed by cell kill and progression delay

If careful attention is paid to detail, these inconsistencies do not seem insurmountable. The explicit inclusion of such biological processes extends the earlier work of Shackney (1970) and Himmelstein and Bischoff (1973).

The simulation of Bhuyan's *in vitro* measurements of ara-C cytotoxicity is shown in Figs. 6, 7, and 8.

Figure 6 shows the dose response obtained by Bhuyan, together with our simulation. In Bhuyan's dose-response tests, cells were exposed to ara-C for one hour, washed, and then subjected to a plating assay to determine cell survival. But, because of the long half-life for decay of ara-CTP, and because the nucleotide must be dephosphorylated before it leaves the cell, cell washing does not terminate the effect of the intracellular levels of ara-CTP. This is a consistent theme of our simulations. A one hour *in vitro* exposure to ara-C is equivalent to a much longer exposure to ara-CTP, the exact length depending on kinase levels and ara-C levels.

Figure 7 compares the simulated survival curve for a continuous exposure to 20 μ M ara-C with the data of Bhuyan. The set of biochemical

Fig. 6—Dose response curve: simulation of Bhuyan's measurements, *in vitro* exposure of L1210 cells to ara-C for one hour, and then washing and plating of cells

Fig. 7—Simulation of Bhuyan's (1973) measurements, ara-C single-dose survival curve *in vitro*, 20 μM ara-C concentration for different times of exposure, and then washing and plating of cells

Fig. 8—Simulation of Bhuyan's (1973) measurements, ara-C divided dose *in vitro*, *in vitro* exposure of L1210 cells to ara-C at a concentration of $20\mu\text{M}$ for one hour; then exposure, after various time intervals, to a second dose at the same level; then washing and plating of cells. The simulation, in this instance, is very sensitive to both ara-CTP level and progression delay

cell-kinetic coupling parameters described in Eqs. (11)-(14) are chosen on the basis of qualitative agreement with Figs. 6 and 7.

Figure 8 presents the results of Bhuyan's split-dose experiments and our simulation. The parameters were identical to those used in the simulations shown in Figs. 6 and 7. The key observation which follows from this simulation is that kill due to the second dose is extremely sensitive to the intracellular half-life of ara-CTP. If the half-life is long, the L1210 cells remain blocked in the very early part of S-phase where the values of the sensitivity function $F(\mu)$ are low (see Fig. 4). They do not progress into the more lethal mid-S-phase until much too late to account for the position of the first minimum in Fig. 8 (the time of maximum kill due to the second dose). The simulation reproduces the data of Bhuyan relatively well, but shows a slight delay in the onset of maximum kill. A higher deoxycytidine kinase activity and 50-min ara-CTP half-life could also account for this split-dose data, but such a short half-life would be inconsistent with the DNA synthesis recovery data of Chou.

The consistency of our simulations with available data for the pharmacokinetic disposition of ara-C and its intracellular metabolites has already been described (Morrison et al., 1975).

The response of L1210 cells growing in the femoral marrow of leukemic mice to single injections of ara-C was simulated in order to compare the model with the experiments by Edelstein et al. (1974). Figure 9 shows the simulation of a dose of 1 mg/mouse. This exposure is roughly equivalent to the *in vitro* work of Bhuyan. The simulations were carried out in two stages. In the first stage, the ara-CTP concentration on the marrow of a mouse infiltrated with L1210 cells was simulated, and this is shown in Fig. 9. Two changes from the earlier simulations have been made. First, the competition of 1mM AMP for the 5'-nucleotidase enzyme has been included in the kinetic expression for the phosphatase. Second, the nucleotide ratios of ara-CTP and ara-CDP to ara-CMP have been reassigned to equal 7.1 and 1.6, respectively, agreeing with Chou's (1975) measurements for the L1210 system. These two changes appear to be self-canceling, since the computer half-life of ara-CTP is unchanged from its earlier value.

In the second stage, the ara-CTP values were used in Eqs. (6) and (11) to determine the corresponding cell-surviving fraction to that measured by the spleen colony assay used by the Washington University group. To obtain simulation of the experimental data, with this degree of precision, it was necessary to adjust the activity of the deoxycytidine kinase. In this instance, Ho's value of V_k of 16.6 $\mu\text{M}/\text{min}$, obtained from cell extract, yielded a predicted ara-CTP time history which provided the appropriate cell-killing effect. Our additional simulations of other doses are shown in Figs. 10 and 11. Figure 10 corresponds to a low dose of .1 mg/mouse, and our simulation shows that additional cellular lethality after the initial killing of S-phase cells is negligible. Cellular progression is delayed for 15 hours, blocked cells rapidly desynchronize, and the growth rate quickly assumes its exponential (asynchronous) value. It is difficult to compare the simulation with the scattered data in this instance. Qualitatively, there is similarity, but the experiments show slight but pronounced reduction in viability until the time of significant regrowth. These are the levels which roughly correspond to clinical doses of ara-C.

Fig. 9—Ara-C effects on L1210 *in vivo*, 1 mg/mouse. The simulation corresponds to Edelstein's (1975) measurements, where 2×10^6 L1210 cells are inoculated i.v. into C3D2F₁ mice, and a single i.v. injection of ara-C is given on day 4. At various times after the ara-C injection, the femoral marrow of the mice is removed, and the number of leukemic colony-forming units (LCFU) is determined using the spleen colony assay. Simulated levels of ara-CTP are also shown. Significant inhibition of DNA synthesis exists for 14 hours, by comparison with Fig. 5

Fig. 10—Ara-C effects on L1210 *in vivo*, .1 mg/mouse. The simulation corresponds to Edelstein's (1975) protocol outlined in Fig. 9. At this dose, which is comparable to levels achieved during clinical treatment of AML, the primary effect after the initial cell killing is to delay regrowth of cell survivors. The simulated levels of ara-CTP suggest that appreciable inhibition of DNA synthesis exists for 12 hours, by comparison with Fig. 5

Fig. 11—Ara-C effects on L1210 in vivo, 10mg/mouse. The simulation corresponds to Edelstein's (1975) experiments outlined in Fig. 9. At this high dose, the simulation predicts that the short-exposure surviving fraction is less than 10 percent. Cells that were in G₁ and G₂ during initial exposure progress into a sensitive phase while ara-CTP levels are still high. The peak ara-CTP level with L1210 cells growing in marrow is comparable to that achieved by Skipper during his effective single i.p. injection treatment of L1210 ascites cells growing in mouse peritoneum

The high-dose simulation (Fig. 11) is for 10 mg of ara-C per mouse. In this case, again, there is reasonable agreement between predicted simulation and experimental measurement. An interesting feature of this simulation, and of the data, is that the effect of a high dose of ara-C, even at very short times of exposure, is to kill many more cells than are in S-phase. For example, our predicted value of short-exposure surviving fraction is less than 7 percent of the cells. This suggests that most of the cells that were in cell-kinetic sanctuary at the time of injection (G_1 , G_2 phases of cycle) were ultimately killed by the lethal amounts of ara-CTP remaining within the cell, even after the L1210 cells had been removed from the host animal. This is consistent with the data, and implications are described in Sec. V. This dose level approximates the exposure of L1210 cells to ara-C using the intraperitoneal route in Skipper's best pulse-dose protocol.

V. DISCUSSION

In this report we have undertaken a difficult problem in quantitative biology: to simulate (1) the pharmacokinetic distribution of cytosine arabinoside to tissues; (2) the intracellular biochemical kinetics of ara-C phosphorylation, dephosphorylation, and deamination; and (3) the detailed effects of this drug on the proliferation cycle of a growing tumor. This integrative approach poses the following questions: Do we understand these components well enough to model them individually? And do we understand them well enough to integrate them?

At present, we would answer these questions with a cautious yes. The questions, however, must be considered on two levels.

One level is intimately related to the data at hand: How should data about the same system be compared when the results stem from different laboratories using different methods, different animal or tumor strains, or when investigators take different measurements?

The other level poses the problem of extrapolation and biological generality: How should the specific results of these particular biological experiments be used to solve the complex problems of clinical treatment?

The first level has already been considered, in part, in Sec. IV, because simulation is a form of experiment designed to produce *more* than curves that fit. We identify the areas of uncertainty that could be clarified by a few additional measurements. These include the measurement of enzyme activities that control ara-CTP level and half life: deoxycytidine kinase and phosphatase, and the ara-CTP levels over time in tumor cells as a function of ara-C exposure. Renal excretion of ara-C and ara-U in the treated tumor-bearing mouse, plus some simple experiments on changes in clearance as a function of water load corresponding to the volumes of saline used as a vehicle for ara-C, would clarify the comparison of *in vivo* experiments. Cell-kinetic measurements giving the total cell number as well as the surviving fraction, together with measures of metabolic viability, would differentiate

between the loss of replicative ability, cell death, and reversible quiescence.

The simulations show the importance of the enzyme activities that determine the level and the half-life of ara-CTP. We can show by simulation that if the half-life of ara-CTP is held constant for all experimental L1210 data as well as for the cell-kinetic parameters, and if the cells are exposed to the appropriate measured or pharmacokinetically calculated dose of ara-C, then only the deoxycytidine kinase activity has to be varied according to the laboratory source of the L1210 cells in order to accurately reproduce and predict the experimental survival data.

Thus the experimental question is raised: Do these L1210 cells vary from one another in their kinase activity? A determination and comparison of the deoxycytidine kinase activity and ara-CTP half-life in the L1210 cells from the laboratories of Bhuyan, Chou, and Valeriote would resolve this question.

Variations in kinase activity in cells maintained in ara-C-containing medium lead to the growth of resistant low-kinase cell lines. This variation and selection is easily accomplished because deoxycytidine kinase is a salvage enzyme not on the main metabolic pathway to DNA synthesis. Even much less directed selection pressures and differences in kinase levels may be expected to develop. The ara-CMP phosphatase enzyme has not been found to change its activity even in cells grown in ara-C-containing medium (Schrecker, 1970). Thus the activity of this enzyme is a less likely candidate for variations among laboratories.

If differences in enzyme activities (or renal clearance) do not account for the differences between tissue culture and *in vivo* results, then the hostility of the tumor cell environment must be taken into account. Host defense mechanisms could enhance the cytotoxic effect of a given level of ara-CTP so that the impact of the drug would be greater *in vivo* than *in vitro*. For example, macrophages might attack and remove sub-lethally damaged cells before they had a chance to recover,¹

¹*In vitro* experiments to clarify the effect of activated macrophages on ara-C dose response could be performed using a modification of techniques of Holtermann et al. (1975).

or a tenuous balance between tumor growth and tumor-cell immunity might be tipped in favor of the host by increased cycle times due to progression delay.

At the more general level, simulations that interpret particular experiments shed light on some of the broad issues of cell kinetics and chemotherapy. As a matter of convenience, we label these issues with the name of the investigator who has posed the problem most clearly:

1. *Steel's Dilemma* (Steel, 1974a, 1974b)
Why is it that cell-kinetic arguments derived from thymidine labeling experiments do not predict the chemotherapeutic effects of cycle-sensitive agents?
2. *Hartmann's Puzzle* (Dombernowsky and Hartmann, 1972; Dombernowsky et al., 1973)
How should the changing cell kinetics of growing tumor populations be modeled to consistently account for all of the data?
3. *Rubinow's Paradox* (Rubinow, 1968; Aroesty et al., 1973)
How should the cell-kinetic relatedness of daughter cells to parent cells be introduced so as to satisfy both the short-term and long-term experimental results of synchronized cultures?
4. *Skipper's Hypothesis* (Skipper, 1964; Skipper et al., 1970)
How can the experimental success of pulse-dose ara-C therapy in the L1210-bearing mouse and the partial clinical success of the parallel therapy for AML in man be compared now that it is known that the rates of tumor growth are not comparable and that the mechanisms of action of ara-C are significantly different from those assumed by Skipper's explanation?

It will be seen that all of these issues are more or less inter-related. They all depend on the capacity of a model that has been used to interpret one set of data to be extended systematically to interpret additional kinds of experiments, and to be corrected for changes in mechanism as the experiment has improved.

STEEL'S DILEMMA

Steel has drawn attention to two apparently serious inconsistencies in the cell kinetics of chemotherapy which challenge some of the useful simple hypotheses of an earlier time. He presents data and simulations on an impressive number of cell systems.

One inconsistency is experimental, and challenges the premise that the passage through S-phase as defined by continuous labeling with tritiated thymidine is equivalent to the lethal effects of high concentrations of ara-C on DNA synthesis. If these were equivalent, then the curve for all survivors as a fraction of the total time of exposure to ara-C would be the same as the curve of unlabeled cells after an equivalent exposure to tritiated thymidine. This is not the case. More cells are killed than are labeled--which does not lend itself to easy explanation. The second inconsistency concerns the extension of models that are designed to interpret percent labeled mitosis curves to include the phenomenon of continuous labeling.

Steel's data on these same cell systems demonstrate that his cell-cycle models--SAB1, SAB2, and SAB3--which are based on PLM data taken from flash-labeling experiments (Steel and Hanes, 1971) do not predict the cumulative labeling with TH^3 nor the cumulative cytotoxic effect of ara-C. The models underestimate both labeling and cytotoxicity in example after example. The failure of all three models, which offer different hypotheses for nonproliferating pools and cell death, underlines the observation that these concepts are not sufficiently flexible to "correct" his model.

He concludes (Steel, 1974b) that "the thymidine labeling data poorly predict the response of clonogenic tumor cells (to cytosine arabinoside), and that the (SAB) models are even less 'useful.'"

These pessimistic conclusions draw our attention to the need for a deeper understanding of the action of ara-C and point to the need for a more consistent modeling framework.

In the work described here, we can in large measure resolve the inconsistency between TH^3 labeling and ara-C cytotoxicity for the L1210 system. Survival fractions *in vivo* and *in vitro* are measured at the time of transfer of cells to an ara-C-free medium after exposure.

However, the intracellular phosphorylation of ara-C forms a pool of ara-CTP which is phosphorylated relatively slowly with a half-life of at least 90 minutes. Thus cells that have been exposed to sufficiently high doses of ara-C and that are not in S-phase will contain lethal amounts of ara-CTP, which will continue to kill cells as they attempt DNA replication. Given present experimental data and a self-consistent model of ara-C metabolism, we have shown that the ara-CTP pool can extend the effective exposure time by several hours. This is sufficient time to reestablish consistency between thymidine labeling and cytotoxicity, at least for the rapidly growing L1210.

Steel's inconsistency may in fact be associated with clonogenicity in other systems he cites besides L1210. However, our results suggest that pharmacokinetics and intracellular biochemistry are important factors in resolving the inconsistency.

HARTMANN'S PUZZLE

Dombernowsky and Hartmann (1972), Dombernowsky et al. (1973), and Jansson (1972, 1973) have set themselves a complex modeling task which we call Hartmann's Puzzle. They consider a cell population growing in a limited environment. As the population density increases, overall growth slows down and the cell-cycle kinetics of the population changes. These investigators utilize four kinds of data at three different cell densities: (1) a flash-labeling index using TH^3 , (2) a PLM curve, (3) a cumulative labeling index, and (4) a distribution of DNA content in a sample population.

The puzzle is to construct a model that will be consistent with the above data not only at one cell density but throughout the growth of the population. We complicate the puzzle by the additional caveat that the same model structure (with different parameter values) should be applicable to different cell types. Data on L1210, the JB1, and the S180 are available.

For these three tumors, Dombernowsky and Hartmann propose three separate models, patterned after those of Steel. As in Steel's experience, the continuous labeling index (CLI) gives a poor fit to each tumor, although initially quiescent cells can be introduced to improve

it. Even ignoring this difficulty, the same model structure does not apply to all tumors. This inconclusive attempt adds further weight to the observed limitations of Steel's modeling approach. Steel's approach and ours may both be used to interpret PLM curves. However, as in Hartmann's Puzzle, where the consistency of more and more data is demanded, differences in the detailed description of cellular flow become important. We have obtained a reasonable fit to the data in Hartmann's Puzzle while still maintaining a structure that is consistent with intracellular enzyme chemistry. Results for the L1210 and the JBI have been obtained and will be described in subsequent reports, but a brief summary is in order.

For both L1210 and JBI, it was shown that a distribution of cycle times corresponding to a displaced gamma distribution characterized by three parameters--minimum cycle time, mean cycle time, and coefficient of variation--was appropriate for simulation. The coefficient of variation, the fraction of cycle time spent in each phase of the cell cycle, the difference between the mean time of transit and the minimum cycle time, and the sterilization rate (discussed below) were all independent of tumor size. The mean cycle time increased with increasing tumor burden in a way that was consistent with damped (Gompertzian) growth. It should be emphasized that our model structure differs substantially from the standard approach. The maturity intervals corresponding to the different phases of cycle could be approximated by constants, μ_{G_1} , μ_S , μ_M , independent of individual cell cycle time. Thus, the phase¹ durations are highly correlated.

An intriguing hypothesis from our explanation of Hartmann's Puzzle is a simple assumption about cell death which appears valid for the systems tested: some proportion of cells are presumed to be "sterilized" as a function of time, the proportion being a characteristic of the cell time and its environment. These sterilized cells (similar to Steel's doomed cells in concept) live to maturity, when they die instead of dividing. The sterilization rate independent of cycle time may be interpreted as an external influence measuring the "hostility" of the environment. *In vivo*, a proportion of that hostility may be the immune response. This hypothesis is susceptible to test by repeating a

Hartmann's Puzzle experiment in a set of animals partially immunized against a particular tumor and comparing these results to a parallel set that is not immunized. The model parameters for these two cases should differ in one term only--the term for cell sterilization. Increased sterilization should represent increased immunological resistance. Janik and Steel's (1972) results for immunologically perturbed tumors support this position. It was shown that immunologic stimulation had little effect on PLM measurements, but showed more considerable effects on growth rate, implying the primary dependency of the sterilization rate on natural hostility.

Our use of Hartmann's Puzzle is designed to uncover a common cell-kinetic model structure that will fit a broad class of cell types. Differences in cell types should be representable by different sets of parameter values without ad hoc revisions to the structure itself. Thus we would expect to describe acute leukemic cell growth in a kinetic structure which is compatible with that used to describe the L1210, but with parameters appropriate to its slower growth rate and extensions to account for the observed degree of differentiation. Changing growth characteristics which are a function of cell density provide the framework for describing the competition between normal marrow and leukemic marrow.

RUBINOW'S PARADOX

This so-called paradox bears on the question of how the inheritance of cycle times by daughter cells contributes to neoplastic selection and tumor cell resistance. The problem also appears when one attempts to model PLM curves which show waves that extend beyond two cycles.

The paradox can be clearly demonstrated by attempting to interpret the decay of synchrony in a group of synchronized tetrahymena (Rubinow, 1968; Aroesty, 1973). In perfectly synchronized cells with the same cycle times, the population would grow in a large series of steps, each step marking a division and each step twice as large as the last. Where there is a distribution of cycle times, these steps appear as waves which damp out to an exponential curve as the cycle times are redistributed. The problem arises when one attempts to model the

desynchronization that damps out these steps. Like ourselves, Rubinow has investigated the class of models that implies a certain biological consistency in the rate of progression through the cell cycle. A cell is born with a given maturation rate which it keeps until division. A growing cell population is distributed over a range of maturation asynchronously.

At the time of mitosis, cells are reborn with new maturation rates which are chosen to yield a steady-state distribution comparable with experiment. New maturation rates may be assigned in many ways. At one extreme, daughter cells may be assigned to maturation rates that are independent of the parent cell. This random heredity may be constrained by a probability distribution. In this way a steady-state distribution can be continually maintained. At the other extreme, each daughter cell can be assigned a maturation rate exactly like that of the parent. This is the case of perfect heredity. In this situation, rapidly dividing cells outgrow the slow ones after many generation times, and the maturation rate distribution becomes a δ -function centered at the shortest T.

When the decay of synchrony for tetrahymena is simulated using Rubinow's model, and a random redistribution maintains the steady state for tetrahymena cycle times, the measured waves and the simulated waves do not match. Only in the long run does the growth rate reach the appropriate steady-state value. However, if perfect heredity is assumed, the short-term match between the growth wave and the simulation is excellent, but in the long run the steady-state growth rate is that of the shortest cycle time.

Rubinow's paradoxical result serves to show that the von Foerster-type mathematical model poses difficulties that cannot be overcome by a simple boundary condition. Although this problem is hardly approachable using the compartment model system, it can be resolved with the flow model approach. The paradox appears when one is restricted to the two extreme cases of perfect heredity and random redistribution. It is also not solved by making simple proportional combinations of these two functions. An appropriate match requires a more complex set of assumptions: a form of heredity that incorporates a variance from a parent cell to daughter cells, and a bias to account for the drift

of the mean and the maintenance of the steady-state distribution.

It is already known, and our own simulations confirm this, that PLM data can usually be interpreted neglecting heredity. Valleron's (1977) work¹ suggests that there are other instances where heredity can play a significant role even during the short times associated with labeled mitoses data. Long-term exposure to chemotherapy can select cells with more rapid cycle times, but the possible interplay between heredity and enzyme kinetics has not been examined either experimentally or by simulation.

SKIPPER'S HYPOTHESIS

Skipper's Hypothesis is that the treatment of L1210 in the mouse with ara-C is a good pharmacokinetic and cell-kinetic animal model for the treatment of acute leukemia in man (Skipper et al., 1970). He observed that it took a very large single dose of ara-C to achieve a significant level of toxicity (LD10) in the mouse, but that a total dose less than one-seventh as large as one intraperitoneal injection distributed evenly over 16 days produced the same toxicity. The daily dose regimen suppressed the tumor, but did not eradicate it. This same lower total dose distributed in smaller increments every three hours for twenty-four hours and repeated every fourth day yielded the same host toxicity. This pulse dose regimen "cured" the mice.

Skipper sketched a rationale for these results: The Q3h x 8-pulse-dose regimen kept the level of ara-C high enough for about 30 hours so that more than 99.9 percent of the L1210 cells had entered the DNA synthesis and were exposed to lethal cytotoxic effects. Thirty hours included all but the tail of the distribution of L1210 cycle times while the host tissues were protected by the survival of low-growth fraction cells present in normal tissues. These cells passed through a drug-free window in the three days between courses. (In the daily regimen, a drug-free window occupying a portion of each day was equally good for the host, but also provided a window for relatively short-cycle-time tumor cells.) The three-day interval was too short for the

¹Described at the NCI Conference on Cell Kinetics and Cancer Chemotherapy, November 4-6, 1975, Annapolis, Maryland.

L1210 cells to recover their previous population, so that in a sequence of courses, all tumor cells were destroyed by the stepwise application to cell kill. This rationale suggested pulse-dose treatment schedules for acute myelogenous leukemia (AML) chemotherapy in man, typified by the pulse-dose COAP regimens (Hart et al., 1969). These were remarkably successful in achieving remission, but unlike the mouse regimen have not eradicated the last proliferating cells in the human leukemias (Freireich et al., 1971). Moreover, in man, continuous intravenous ara-C at 100 mg/meter^2 for five to seven days has proved to be as effective as pulse-dose therapy Q8h at the same dose per day without increased toxicity (Freireich, 1974).

Clarkson et al. (1970) showed by classical labeling experiments that AML cells in man grow more slowly than normal cells. Thus, Skipper's formal argument as applied to man is undermined from both sides. Differential kill based on rapid growth was not a valid rationale, and the rationale for host toxicity could also not be supported.

Skipper's Hypothesis runs into further problems because the biochemistry is incomplete. As more information about the pharmacokinetics and metabolism of ara-C in mice and L1210 has been obtained, it has become clear that the reasons for Skipper's initial success are considerably more complex than originally supposed. Ara-C is converted within cells to a longer-acting active form, ara-CTP, which is cytotoxic at high intracellular levels and cytostatic at lower levels. These lower cytostatic levels "block" DNA synthesis causing progression delay (Chou et al., 1975; Bhuyan et al., 1973). The effect of multiple doses of cytosine arabinoside on cells in the proliferative cycle can only be quantified if these features are taken into account. The fruitfulness of Skipper's Hypothesis needs no defense. Our present understanding of ara-C both in the clinic and in the laboratory is in large measure the result of his proposal and the work it engendered.

It is clear from the work of Bhuyan and Edelstein that a high dose of ara-C causes a limited cell synchrony. First, cells in S-phase are killed. Cells entering S-phase continue to be killed until the concentration of ara-CTP falls to a level where there is only progression delay. For an additional period of time, cells pile up at the beginning

of S-phase so that after this block is released there is an increased proportion of cells in S-phase and an increased susceptibility to a second dose. Divided dose experiments quantify this result. These phenomena are important in the Skipper L1210 experiment.

High dose alone has a very exaggerated effect on transplanted tumors. Our simulations of the Edelstein experiments on L1210 leukemia growing in murine femoral marrow predict that 80 percent of the cells that are not in S-phase at time of exposure die when the animal is injected with a 10-mg dose, even if they are removed from host marrow within an hour.

For intraperitoneal injection of ara-C into animals bearing intraperitoneal L1210 cells, this effect is more pronounced. The initial high levels of ara-C in the cavity are rapidly phosphorylated to higher levels of intracellular ara-CTP within ascites cells than if the same dose is given intravenously or subcutaneously; thus even more tumor cells are killed. Skipper achieves an often unrecognized therapeutic advantage in this manner.

- The success of Skipper's protocol in the main would appear to depend largely on pharmacokinetic advantages achieved by route of administration and the long-term presence of ara-CTP. A cell-kinetic enhancement of these progression delays can also be demonstrated.

Although the same biological processes are operative in AML, the situation is so different that the same explanation cannot hold. In Skipper's initial hypothesis he compared low cell densities in mice to high cell densities in man.

Pulse-dose therapy with ara-C is effective in mice against the proliferation of L1210 at both high and low cell densities. In man, pulse-dose therapy appears to be effective only in the overt AML where the density of leukemia cells is high and the cell population is growing slowly, probably due to a lengthening of cycle times and to an increase in the death rate.

Thus the paramount question becomes: How does cytosine arabinoside in pulse or continuous dose exhibit its effectiveness in the slow growing AML in relapse? Here results are measured by a striking loss in tumor mass, but there is a diminution of therapeutic advantage in the tumor cell density decrease so that no more than a remission marrow is possible.

We can at least propose an answer to this question which lies within the scope of our simulations. It places much more weight on the differential advantage of progression delay than on that of cell kill.

In man there appears to be little pharmacokinetic advantage. We have shown elsewhere (Lincoln et al., 1974) that ara-C intravenous push offers an advantage in tissue distribution due to flow considerations, but that this advantage is virtually lost when the drug is given as a 15-minute infusion. There is no significant difference between these modalities, or indeed between pulse dose push and continuous 5-day infusion. Normal hematopoietic tissue would appear to contain sufficiently high values of kinase (Ho and Frei, 1971) to offer no protection. Differences in the level of deaminase do not appear to be as significant as once supposed.

The simplest cell-kinetic proposal states that while both normal cells and tumor cells are killed by the drug, the more rapidly growing normal cells outgrow the slower growing tumor cells and reestablish a remission marrow. This advantage may be lost as the cell density of tumor cells falls and their cycle-kinetic times grow shorter to approximate those of normal cells. Rubinow has proposed a model that incorporates this instability in a hematopoietic control system.

Our models point to an additional factor that depends on cell loss. Moore et al. (1974) have pointed out that the effectiveness of cycle-sensitive drugs in inducing remission in AML appears to be related to a high instability in the tumor population characterized by a high cell loss rate in agar culture.

If there is a cell loss via death or end-stage cells which is independent of maturation rate, then this loss will be enhanced by progression delay which will significantly slow the passage through the cell cycle. Such a cell loss might depend on the inherent fragility of a tumor population, but it might also depend on the hostility of the tumor environment. This hostility might be nothing more than a lack of nutritional components or the presence of certain toxic metabolites, or it might be the exposure to other drugs such as Cytoxan or vincristine, or it might involve host reactions including host tissue immunity. For any of the above cases, the immediate result of progression delay

would be a loss of tumor mass with the reestablishment of a new equilibrium at a lower level.

Only in the case of tissue immunity might a permanent advantage be gained. An enhanced immune response would increase the difference in loss rate between tumor cells and normal cells, facilitating remission and leading, in some cases at least, to the elimination of the last tumor cell. Only if a tissue-immune response against leukemic cells were present would an advantage be retained when the cell cycle is short. This response would be enhanced if the cycle time is prolonged by cytostatic doses of ara-C in the patient in remission. This may be the key to remission maintenance, which can be sustained with BCG-immune enhancement or with intermittent doses of agents such as ara-C as long as the patient exhibits tissue immunity (Gutterman et al., 1974). Moreover, in patients treated with chemotherapy and immunotherapy who subsequently relapse, limited experience indicates (Freireich, 1974) that a second remission is easier to achieve than in the cases that had received no immune enhancement.

The interaction among these mechanisms and their detailed relation to cell kinetics have not yet been modeled for lack of experimental data, although Janik and Steel's (1972) work is highly suggestive.

Our approach to cell-cycle kinetics has been to include the variation in maturation rates associated with different cells, which will allow us to consider most of the above phenomena. However, we still neglect any intercellular variation in enzyme kinetics or nucleotide values. This is primarily because variation in maturation rate is the key to understanding labeling mitosis data, while biochemical and pharmacologic measurements are given as mean values, in the main. Experimental or clinical protocols involving synchronization or recruitment strategies may be sensitive to such variations. Presently, our simulations show desynchronization after a period of progression delay which is due to variation in maturation rate (cycle-time distribution). Other mechanisms for desynchronization may in fact be operating.

VI. CONCLUSIONS

To accurately interpret the cell-kinetic *in vivo* chemotherapy experiments such as those of Skipper or Edelstein and to relate them to man, organ pharmacokinetics, intracellular pharmacology, and the proliferation kinetics of tumor cells must be integrated to expose the underlying biological processes that are brought into play. This particular structure appears to be an intricate and complicated way to organize our thoughts. Ultimately, an analysis of sensitivity may lead to simpler formulations, but for the present it is the only way to capture the subtlety needed for prediction and quantitative therapeutic manipulation.

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