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MODELS FOR STATISTICAL ANALYSIS OF EXPERIMENTS USING REPEATED DRUG TREATMENTS: CHEMOTHERAPY OF L1210 LEUKEMIA IN MICE

by

Nelly Szabo Ullman

A dissertation submitted in partial fulfillment
of the requirements for the degree of
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ABSTRACT

MODELS FOR STATISTICAL ANALYSIS OF EXPERIMENTS
USING REPEATED DRUG TREATMENTS:
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by

Nelly Szabo Ullman

Chairman: John A. Jacquez

Multiple applications of a drug are often used in chemotherapy. The analysis of a strategy of drug application should take into account kinetic processes occuring in the host and in the disease bearing organisms. The growth of these organisms and retention of drug in the host are important factors to be considered. In this thesis, a basic model and variations of it, which take these and other factors into account, are set up for analyzing multiple dose chemotherapy experiments. It is assumed that the disease bearing organisms vary in susceptibility to the drug, the response appearing in the models as a tolerance distribution.

The models are applied to experiments on L1210 leukemia in mice by Skipper, Schabel and Wilcox (Cancer Chemotherapy Reports 35:1964). The drugs used in these experiments were amethopterin and NSC 409962. In the process of analyzing these experiments by any of these models, a set of nonlinear equations in three or more parameters is obtained. The parameters appearing in all models treated are drug retention, and the mean and variance of the tolerance distribution. Another

parameter common to all models, the rate of leukemic cell growth, is derived from other experiments.

The modifications of the basic model that were examined and applied were: 1) appearance of a drug resistant line of leukemic cells, 2) toxicity of drug for the host, 3) weakening of a population after repeated dosing, and 4) time delay of drug effect. Each of these variations required the introduction of at least one more parameter.

Parameters were evaluated from the experiments using a least squares method. The equations are nonlinear. Several methods were used in the minimization process, the most practical being a "direct search" procedure.

The results show that NSC 409962 is not stored effectively in the host during treatment. Amethopterin is stored in the mouse, but a simple application of the model makes it appear as if the drug is stored in excess of the amount applied. It is probable, but not certain, that amethopterin is metabolized to a more effective form, and that a more precise picture of action must take into account the transmutation of amethopterin in the host.

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TABLE OF CONTENTS

		Page
INTRODUCTION		1
1.	Statement of the Problem	1
2.	Leukemia	4
3.	Chemotherapy Experiments on L1210 Leukemic Mice	7
4.	Resistance to Drug	8
5.	Models of Cell Growth	9
6.	Characteristics and Mode of Action of a Drug	16
7.	Models of Drug Action	19
Chapter		
I.	THE MODELS	24
	I. Introduction	24
	II. Properties of the Models	25
	III. Description of the Models	28
	IV. Summary	34
II.	STATISTICAL ANALYSIS	35
	I. Introduction	35
	II. Criteria for Best Fit	35
	III. Minimization	38
III.	RESULTS OF DETERMINATION OF PARAMETERS	44
	I. Introduction	44
	II. NSC 409962	44
	III. Amethopterin	63
IV.	DISCUSSION PART I - GENERAL	75
V.	DISCUSSION PART II - AMETHOPTERIN	81
	I. Introduction	81
	II. A Simple Continuous Model	82
	III. Search for An Explanation of Negative β in Amethopterin Experiments	87

Chapter Page

VI. CONCLUSION. 98

APPENDIX A 102

APPENDIX B 104

LIST OF REFERENCES 109

LIST OF TABLES

Table		Page
1.	Data Set I - NSC 409962	47
2.	Parameters Obtained for NSC 409962 - Data Set I By Minimization	46
3.	Values of N_k, R_k, Log R_k, Calculated Minus Observed Day of Death for NSC 409962 Experiments of Data Set I, Cell Model I, Using Parameters Listed in Table 2.	49
4.	Values of N_k, R_k, Log R_k, Calculated Minus Observed Day of Death for NSC 409962 Experiments of Data Set I, Cell Model IR, Using Parameters Listed in Table 2.	50
5.	Data Set II - NSC 409962.	53
6.	Parameters Obtained for the Minimum Point and At A Neighboring Point NSC 409962 - Data Set II - Cell Model I	54
7.	Parameters Obtained for the Minimum Point and At A Neighboring Point NSC 409962 - Data Set II - Cell Model IR.	54
8.	Values of N_k, R_k, Log R_k, Calculated Minus Observed Day of Death for NSC 409962 for Data Set II, Cell Model I, Using Parameters At Minimum Point Listed In Table 6	56
9.	Values of N_k, R_k, Log R_k, Calculated Minus Observed Day of Death for NSC 409962 for Data Set II, Cell Model IR, Using Parameters At Minimum Point Listed In Table 7. . .	57
10.	Data Set II* - NSC 409962 - Consisting of All Experiments In Data Set II Whose Total Dose Is Less Than 88 mg/kg.wt..	60
11.	Parameters Obtained for the Minimum Point and At A Neighboring NSC 409962 - Data Set II* - Cell Model I.	59

Table | Page

12. Values of N_k, R_k, Log R_k, Calculated Minus Observed Day of Death for NSC 409962, Data Set II*, Cell Model I, Using Parameters of Minimum Point Listed in Table 11. 61

13. Values of N_k, R_k, Log R_k, Calculated Minus Observed Day of Death for NSC 409962, Data Set II*, Cell Model I, Using Parameters of Neighboring Point Listed in Table 11. 62

14. Values of N_k, R_k, Log R_k, Calculated Minus Observed Day of Death for NSC 409962, Data Set II, Cell Model I, Using Parameters Fitted to Low Dose Points Listed in Table 11 as "Minimum Point". 66

15. Values of N_k, R_k, Log R_k, Calculated Minus Observed Day of Death for NSC 409962, Data Set II, Cell Model I, Using Parameters Fitted to Low Dose Points Listed in Table 11 as "Neighboring" Point. 67

16. Amethopterin Data 69

17. Parameters Obtained for Amethopterin Data Listed in Table 16 by Least Squares Minimization, Cell Model I. 68

18. Values of N_k, R_k, Log R_k, Calculated Minus Observed Day of Death for Amethopterin - Cell Model I, Using Parameters Listed in Table 17. 70

19. Parameters Obtained for Amethopterin Data Listed in Table 16 by Least Squares Minimization Using $\alpha = \alpha_0 \exp(-\Gamma Z_k)$, $\alpha_0 = 1.53506$. 88

20. Parameters Obtained for Amethopterin Data Listed in Table 16 by Least Squares Minimization Using $\mu = \mu_0 \exp(-\delta Z_k)$. . . 88

21. Parameters Obtained for Amethopterin Data Listed in Table 16 by Least Squares Minimization Using "Delayed Drug Action". . . 92

22. Comparison of Effective Dose at Time of Each Treatment for "Delayed-Drug Action" Variation of Cell Model I (Using Parameters of Table 21 and for the Original Cell Model I (Using Parameters of Table 27) $Z_0 = 1$ mg/kg.wt.. 93

Table | Page

23. Values of N_k, R_k, Log R_k, Calculated Minus Observed Day of Death for Amethopterin Using "Time-Delay" Model and Parameters Listed in Table 21. 94

24. Comparisons of Minimization Methods Described in Chapter II, Section III-B on Data Generated with Model with Surviving Parent Parameters Listed in Appendix B 106

25. Comparison of Different Starting Points and Outcome in Direct Search Minimization Method Using Generated Data α is Fixed at 0.4, β is Fixed at 0.8. 108

LIST OF ILLUSTRATIONS

Figure		Page
1.	Dose vs. Time	30
2.	Number of Survivors (N) vs. Time.	30
3.	A Plot of NSC 409962 Data Set I Using Parameters of Table 1 for Cell Model I. . . .	51
4.	A Plot of NSC 409962 Data Set I Using Parameters of Table 1 for Cell Model IR . . .	52
5.	A Plot of NSC 409962 Data Set II Using Results of Tables 8 and 9	58
6.	A Plot of $Log_{10}{}^{R}$ vs. Total Dose. NSC 409962-Data Set II* Using Parameters of Minimum Point Listed in Table 11-High Total Dose Points of Data Set II Added	64
7.	A Plot of $Log_{10}{}^{R}$ vs. Total Dose. NSC 409962-Data Set II* Using Parameters of "Neighboring" Point Listed in Table 11-High Total Dose Points of Data Set II Added	65
8.	A Plot of Log R vs. Total Dose for Amethopterin Data Using Cell Model I and Parameters of Table 17	71
9.	A Plot of μ vs. β for Amethopterin Data of Table 16 Using Cell Model I and the Least Square Criterion Q_5. These are Best Values of μ (and σ^2) as Functions of β With α Fixed at 1.53506.	72
10.	A Plot of Q_5 vs. β for Amethopterin Data of Table 16 Using Cell Model I. The Q_5 Value is that Obtained with the Best Values of μ and σ^2 as A Function of β, With α Fixed at 1.53506.	73

Figure Page

11. Sketches of Population Size vs. Time. 78

12. In Growth vs. Time for $\beta = 0$. Continuous Model. Amethopterin Data 86

13. Effective Dose vs. Time for Amethopterin Data (Table 21) 95

14. A Plot of $Log_{10}R$ vs. Total Dose for Amethopterin Data (Table 23) for "Delayed Drug Action" Model (Parameters of Table 21). . . . 96

INTRODUCTION

1. Statement of the Problem

The treatment and cure of cancer is a major medical problem One of the principle methods of treatment is by chemotherapy. The aim of many experimental studies of chemotherapy is to obtain a clear picture of the biochemical interactions between the drug and the diseased cells. This problem can be made precise by formulation of a suitable mathematical model. It is the purpose of this thesis to produce a mathematical model applicable to some aspects of the treatment of leukemia by chemotherapy.

Extensive experimentation on chemotherapy of leukemia is under way. The experiments are performed by treating leukemic mice with a chemical agent at several dose levels and for varying time intervals (15, 31-35). In this paper we propose a framework or model which permits a statistical analysis of these experiments for evaluation of the therapeudic material. The model is applied to data for the treatment of L1210 leukemia in mice using the drugs NSC409962 and amethopterin.

The emphasis here is on multiple-dose treatments as opposed to single massive doses. The permissible amount, and therefore the effectiveness, of a single dose is limited because of toxicity to the host-animal. A common strategy for

control of cancer is a course of repeated drug application whose effectiveness in killing tumor cells matches or surpasses cell growth between doses. However, studies of drug action using a multiple-dose technique are appreciably more complex than those involving a single application. For example, each dose, when applied to the system, may be augmented by residues of previous doses stored either in the original or partially metabolized form (16). In addition, the selective destruction of the weaker members of the leukemic cell population by previous application of the drug, and the growth of progeny of the survivors means that each successive treatment is applied to a cell population of different composition. Some individual cells born in the course of treatment are resistant to the drug and will continue to proliferate despite treatment.

Successful multiple-dose chemotherapy in man or mouse must match dosage levels and schedules with the generation[1] and doubling[1] times of the malignant cells. Determination of the optimal course of treatment is also influenced by factors, such as drug storage, recovery rate from toxicity, and the fraction of neoplastic cells killed by a given dose.

In order to evaluate the action of drugs whose mode of action upon a complex biological system is not completely known, a class of simplified models has been devised. Such models provide a framework within which experiments can be statistically analyzed. The basic model takes into account

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[1]Definition follows

rate of cell growth, drug retention over a period of time, and varying susceptibility of the leukemic cells to the drug (tolerance distribution[1]). Modifications of the basic model which have been examined are 1) the appearance of drug resistant cells, 2) variable cell growth rates, 3) drug weakened populations, 4) toxicity to the host, and 5) delayed drug action. As a consequence of this study we propose the design of new and more incisive experiments.

SPECIALIZED TERMINOLOGY

1) Doubling Time

The average time required for the viable cells to double in number regardless of the mechanism.

2) Generation Time

Generation time of a cell is the time interval between identical phases of two consecutive mitoses. This is frequently confused with doubling time. Since some cells may have lost the capacity to divide or there is cell loss, the doubling time is usually longer than the generation time.

3) Life Span

Life span of a cell is the time interval which elapses from the time of its formation (last mitosis) to the time of death or cell division.

---

[1] Definition follows

4) Quantal Response

A selected dose of drug is applied to a subject. The subject is then classified as having a characteristic response or not. For example, at a particular time after treatment, the organisms are either dead or alive with no account taken of partial effects. A quantitative response, by contrast, measures the degree of reaction.

5) Tolerance

The tolerance of any subject is defined as the minimum dose just sufficient to produce the characteristic response; lesser doses are completely ineffective. The tolerance of an individual is assumed to be a fixed quantity.

6) Tolerance Distribution

The tolerance for a drug varies from one individual to another in a population. The associated frequency distribution is referred to as a TOLERANCE DISTRIBUTION.

2. Leukemia

Leukemia is a disease characterized by non steady state cell proliferation. Human leukemia is divided into three separate categories: chronic granulocytic leukemia, acute lymphoblastic leukemia, and multiple myeloma. Chronic granulocytic leukemia almost always exhibits a chromosomal abnormality which may arise from a genetic mutation induced in some cases by ionizing radiation. Acute lymphoblastic leukemia occurs frequently in conjunction with an infection and

conceivably originates from a virus. Multiple myeloma exists as a neoplasm of cells of the immunological system. This form of the disease may or may not be caused by a virus (10).

The main objective of medical research on leukemia has been to develop methods and techniques leading to the understanding and treatment of human leukemia. Many experiments are carried out using mice injected with a special strain of leukemia, L1210. Mice are used because of economic reasons (cost, lifespan, etc.) and also because they provide a specific compatibility of tumor to host and are available in genetically pure breeds, thereby making experimental control relatively easy.

However, leukemia in mice differs sufficiently from leukemia in humans that extrapolation of knowledge from mouse experiments to human leukemia must be done with great caution.

a. Human Leukemia.--Cronkite (8) points out that human leukemias might well be different from the animal types. In human leukemic growth, actual proliferation might not be rapid but could be normal or even slow. Doubling times in human leukemia vary from 4 days for the acute form to perhaps 30 days for the chronic type. It seems clear that doubling time in humans is longer than the generation time, signifying that cells are dying and/or that a fraction of the cells produced are non-proliferative. Only during the stage when the disease is relapsing does there appear to be true exponential

growth of cells. Spontaneous remissions of human leukemia have been observed. Thus human leukemia may not generally be characterized by unrestrained exponential growth in which each cell participates in proliferation. Rather, it is likely that substantial fluctuations in growth rates of leukemic cells exist, and that leukemic cells consist of both dividing and nondividing populations much like normal cells.

b. L1210 Leukemia in Mice.--The growth pattern of L1210 leukemic cells in mice consists of a two day lag after transplantation, followed by exponential growth with doubling times of approximately 0.5 days. Mouse leukemia is considered a disease of rapid proliferation of an aberrant cell line. In fact, it has been demonstrated that a single L1210 leukemic cell is sufficient to cause the disease. It is believed that all L1210 leukemic cells contribute to mitosis, and that there are no non-proliferating cells as found in human leukemia (8, 33).

Despite these differences in the proliferation patterns of human and L1210 mouse leukemia, mouse leukemia is used for evaluating chemotherapeutic agents. The elimination of every leukemic cell remains the primary objective in present leukemia therapy in man. It is hoped that the fundamental knowledge gained in quantitative and kinetic experiments on mice (not the numbers or proliferation rates) will provide a basis for the design of clinical trials (35).

3. Chemotherapy Experiments on L1210 Leukemic Mice

Experiments using L1210 leukemic mice have produced a number of facts about the disease (33). In summary, they are:

1) Inoculation of a single viable leukemic cell will cause generalized leukemia and death.

2) The average doubling time of experimental leukemic cell populations is near 0.5 day. The lethal number of L1210 leukemia cells is of the order of 10^9 per mouse.

3) Drug-induced increase in host life span is the result of the killing of leukemic cells. The drug does not produce a lag in cell growth, nor does it lead to a selected neoplastic cell population with a slower growth rate.

4) Under certain conditions (where there is no bone marrow damage causing undue normal cell proliferation) some anti-leukemic agents possess rather marked selective toxicity to leukemic cells in vivo.

5) "Cell cures" of experimental leukemias are achieved by killing the leukemic cells faster than proliferation of the survivors can replace them. Treatment must be continued until every leukemic cell is eradicated, with or without host-immune "assistance", and without excessive toxicity to the host.

In experiments evaluating the effectiveness of a drug in achieving the total kill necessary to cure leukemia in mice, it is vital to gain and apply basic information (in a

"matching" sense) concerning 1) the percent leukemic cell kill per dose of a given drug, 2) the rate of proliferation of leukemic cells surviving a given dose of drug, and 3) the speed at which the host recovers from a sublethal dose (35). Only after these parameters are evaluated can a proper therapeudic regimen be prescribed for the treatment of the host mouse having a limited number of drug-sensitive cells.

4. Resistance to Drug

It has been demonstrated experimentally in a number of systems that cell resistance to a drug originates through spontaneous genetic changes and that the agents used act in a selective manner, eliminating susceptible forms and allowing resistant individuals to reproduce. Law (24) has reported the development of resistance in mouse leukemic cells. These resistant variants grow optimally in mice receiving the maximum tolerated dose of drug. Resistance to a specific drug may be considered a stable and irreversible heritable change.

There is no evidence, according to Law, that the mouse's immunological mechanism can deal with or eliminate a lowered leukemic cell count. Therefore the only rational approach is to attempt to eliminate every leukemic cell by drug kill. But since highly resistant variants have been found even when using the most effective antileukemic drugs it cannot be expected that any one drug will eliminate all the leukemic cells. It is possible, however, that complete eradication can be attained by the use of combinations of drugs which act independently, since leukemic cells which are

resistant to one drug may be susceptible to others. Such an ideal combination is not easy to achieve, thus leukemia therapy in man and mouse presently is aimed at the modest goal of an increased life span, rather than a cure.

5. Models of Cell Growth

Consider a population of cells where the individual may grow and divide, or possibly die without division. If the properties of the individual cells are known, and if the factors governing their growth and division are understood, predictions concerning the development of the population are possible. Many mathematical models have been devised which deal with cell growth. Some of these are deterministic, some are stochastic, and others combine the two approaches. These models generally describe all growth under the assumption of a variety of boundary conditions or frequency distributions, and some of these treat tumor growth.

In many mathematical treatments of a cell system the population is partitioned into compartments ("demes"). Von Foerster (41) defines a deme as consisting of all cell compartments answering a well-defined state-description (such as cells in the S phase with respect to DNA synthesis). If there are n demes, and if $N_i(t)$ denotes the number of cells in the i^{th} deme as a function of time, t, the simplest mathematical model is a set of "balance equations" (38,39), describing the flow of cells through the system

$$\frac{dN_i}{dt} = A_i - B_i \qquad i = 1,2...n. \qquad (1)$$

Here A_i is the total accretion rate, which includes birth as well as immigration, and B_i is the depletion rate which includes deaths and loss to other demes. As long as A_i and B_i $(i = 1,2...n)$ depend linearly on the numbers N_i and on nothing else, the solutions to (1) are easily found. This is the case for populations growing exponentially.

However equation (1) neglects an important feature of the system, i.e. the age distribution within each deme. The age of a cell is the time measured from the instant when the cell acquires all the attributes of the deme (or state) to which it belongs. Age is the simplest variable describing the changes of a cells' intrinsic properties (such as cell size) during its life in the compartment. Each cell is constantly undergoing "maturation" and age measures the stage reached by the maturation process in each deme.

The life span, or maximum age reached by a cell is a variable. For this reason it is improbable that the rates of loss and accretion will continue to depend only on the total number of cells in the deme. Von Foerster (41) generalizes equation (1) by replacing the discrete set of variables n_i by a function $n(t,a)$ where a, the age of the cell, defines the deme. Equation (1) is transformed into

$$\frac{\partial n(t,a)}{\partial t} + \frac{\partial n(t,a)}{\partial a} = -\lambda(t,a,...) \cdot n(t,a) \qquad (2)$$

where λ, the LOSS FUNCTION depends on t,a, and possibly on n, and on other parameters specifying the system. These equations can be solved by specifying certain boundary conditions: $n(t,0) = \alpha(t)$, the generalized birth rate and $\beta(a) = n(0,a)$, the initial age distribution.

Trucco (38) gives a solution of the Von Foerster equation (2) in the special case when λ depends on t (time) and a (age) only. The solution n(t,a) is given in terms of the initial age distribution $\beta(a)$ and the birth rate $\alpha(t)$. He also develops a steady state solution (time-independent) for the case when λ does not depend on n. Trucco treats each generation of cells as a deme so that the birth of new cells is reduced to a zero-th generation coming from a precursor population. For a stem cell compartment, there are no precursors, and the birth rate, $\alpha(t)$, must be determined from the density function n itself. In the case where $\alpha(t)$ is a linear function of the age density n this gives a simple model of a self-replenishing stem cell compartment and leads to necessary conditions for the existence of a steady state. The Von Foerster equations do not deal with the stability of the steady state and the system's response to perturbations.

The models of Von Foerster and Trucco are deterministic. A more refined analysis, which includes the role of chance effects in the development of the population, is provided by stochastic models. A brief description of some of these follows.

Harris (18) deals with a mathematical model for the increase of a population by binary fission. Growth of a population is treated as an example of a random process developing over a period of time whose development is governed by probabilistic considerations. He discusses a type of Markov process useful for certain problems in population growth. He assumes that each cell has a random lifelength T, with a probability density f(t). At the end of its lifetime it splits into two new cells, each of which divides after a lifelength (generation time) that has the same density f(t). He assumes there is no correlation between the lifespan of a mother and daughter, or between sisters. As a consequence, once a cell is replaced by two new ones, no further information, pertinent to the future, is obtained by learning more about the past history of the process. On the other hand, past history is relevant to the behavior of cells selected at an arbitrary moment in time. Suppose that 5 cells are present at some particular instant of time. The subsequent behavior of these cells depends on their ages, i.e. their past history. Thus age of cells plays an important role in predicting the population size both in deterministic and stochastic models of cell growth. Using certain forms of the density function f(t) and this "regenerative" Markov process, Harris sets up equations for the total number of cells as t increases. This stochastic model describes the logarithmic phase of growth well. However once the cells are crowded, the model ceases to be applicable. Harris, himself, suggests

that this later stage of growth is better described by a deterministic model rather than by his probabilistic one. It should also be possible to modify the stochastic model to treat this case. It is also evident that stochastic and deterministic models should lead to the same results if the number of cells is large.

Bell and Anderson (2) have proposed a model for the development of a cell population in which a given cell is characterized by its age and volume. These parameters are assumed to determine the rate of volume growth and the probability per unit time of division or death. Their theory refers to the probability of division or death while a deterministic rate of growth is assumed. The authors discuss the special cases of populations in exponential growth and in the steady state. Bell (1) discusses properties of growth and division which are required for the balanced exponential growth of a cell population. He treats the special case when all cells divide at the same age. He also shows that if the probability of cell division depends on age only, and growth rate is proportional to cell volume, no possibility of balanced exponential growth exists.

Cronkite (8) proposes a theory based on a division of blood forming tissue into three compartments. The first, or stem cell compartment, contains cells with extensive proliferating capacity which have the ability to give rise to new stem cells and to differentiated cells. The second, or

early differentiated cell compartment, consists of cells with limited proliferative capacity which give rise to the third compartment. The latter consists of specialized cells adapted to fulfill specific functional roles. Under normal conditions, the numbers of differentiated cells in the blood remain relatively constant; under conditions of stress or increased demand, however, rapid changes in cell numbers are observed, which are followed by a return to normal levels when the stress is relieved. These features of "hemopoiesis" imply that the production of differentiated cells is under precise control.

The problem of how a differentiated population of cells maintains itself, i.e. how stem cells maintain their numbers after losses by cellular death or by removal of cells is the subject of speculation by three different authors, Osgood (28), Lajtha (23) and Newton (27). Osgood assumes that differentiation occurs by virtue of an asymmetrical mitosis, i.e. a process by which a stem cell divides to produce one differentiated cell and one new stem cell. He postulates that stem cells can also divide symmetrically, producing two stem cells, to replenish the stem cell population after depletion by cellular death. Noting the difficulty of visualizing a mechanism by which cells might shift readily between the two or more types of division, Lajtha proposes the following model: both differentiation and death cause the removal of stem cells from the basic population in which they exist in a state of dormancy. This somehow affects the state of a humoral feedback

control mechanism which triggers[1] the cell cycle and subsequent mitosis to generate the precise number of cells required to restore the population to its normal size. An additional theory, presented by Newton, states that differentiation occurs primarily in maturing stem cells and the probability of a cell differentiating depends both on the cell's level of maturation and upon the concentration of various humoral agents at that time. A cell's response to the agents that induce differentiation is assumed to vary as the biochemical changes which bring it to maturity unfold. Similarly, the probability that the cell will die or that it will embark on mitosis is taken to be a function of cellular maturity, and possibly, of humoral agents.

In conclusion we note that stem cell populations are characterized by self maintainance, i.e. the capacity to maintain their numbers during the life span of the organism in spite of cell loss. Osgood, Lajtha and Newton present different theories of how normal stem cells are prevented from entering into the cell cycle. Abnormal or incomplete operation of such self regulating mechanisms appears to be characteristic of tumor growth.

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[1] This trigger effect presumably operates with a considerable time delay. Trucco tried to introduce this time delay into the Von Foerster model, described earlier in this section, but found that this led to enormous complications. However, a simplified form of Lajtha's model can be derived as a special case of the Von Foerster equations (38, 39).

6. Characteristics and Mode of Action of a Drug

The study of the biochemical and physiological characteristics of drug action focuses on the transport and transformation of the drug as well as its mode of action. These factors, together with dosage determine the concentration of the drug at its site of action, and hence the intensity and temporal course of its effect.

Effective chemotherapeutic drugs destroy parasites, and at the same time injure the host animal only minimally, if at all. This selectivity is one of the most important characteristics of a drug. To produce the required result, a drug must achieve adequate concentrations at its site of action. The concentration is a function of the amount of drug applied, the extent and rate of absorption, distribution, binding or localization in tissues, inactivation and excretion. This progression of steps depends on the transport of the drug across cell membranes. The transport may occur by passage through pores in the membrane. More often it takes place by solubilization in the membrane, diffusion and subsequent desorption from the other side of the membrane.

The rate of absorption largely determines the time interval between administration and effect of the drug. This rate depends on the drug solubility which is determined by local conditions (such as the pH) at the site of absorption. The drug concentration, blood circulation to the site of absorption, and the route of administration all influence the rate of absorption.

After a drug is absorbed, it is distributed in the body by entering or passing through the various body fluid compartments: plasma, interstitial fluid, transcellular fluid and cellular fluids. Some drugs accumulate in special areas as a result of chemical binding, dissolving in fat, or by active transport. This accumulation may either be at the site of action or at a "storage depot". Although the drug in the storage depot may be pharmacologically inactive, it is in equilibrium with free drug in the plasma and thereby maintains an effective concentration of the drug at the site of action. It is released into the plasma, as the drug is metabolized, accumulated in other tissues, or excreted. As a result, effective plasma levels and resulting therapeutic effect of the drug may be maintained for long periods of time.

Although termination of the effect of a drug is usually accomplished by biotransformations[1] and excretion, it may also result from redistribution of the drug from its site of action into other tissues, where it is stored in inactive form, and its ultimate elimination is still dependent on biotransformations and excretion. If the initial dose saturates the storage site, a subsequent dose of the drug may produce a prolonged effect.

Too little is known of cellular biochemistry and physiology to permit other than incomplete or superficial

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[1]Many drugs undergo "biotransformation" which usually results in inactivation of the drug. To be excreted more rapidly (without being reabsorbed in the renal tubes) these drugs are transformed into metabolites which are less able to bind to plasma and tissue proteins, less likely to be stored in fat deposits and less able to penetrate cell membranes.

explanation of the mechanism of action of most drugs. The drug-cell interaction alters some functions of the cell and thereby causes changes which identify and characterize that drug. A drug may produce this effect directly or by changing the action of another substance. Unfortunately this information is rarely available.

Drugs may influence any of the steps involved in the maintainance of normal cell function. Thus they may enhance or prevent the entrance into the cell of substances necessary for energy production or maintainance of osmotic or electrical properties of cells or interfere with mitosis. Drugs may act by influencing the bound forms of endogenous, physiologically active substances. For example, the pharmacological effects of some drugs have been traced to the displacement of hormones from their binding sites on plasma proteins. Drugs may affect the physiochemical property of a cell in an as yet undetermined manner (for example, by forming hydrate crystals with cerebral fluids and thus affecting the electrical property of the brain). For further and more extensive discussion of the characteristics of a drug and its mode of action, see Goodman and Gilman (17).

In a broad sense the basic problems in chemotherapy of neoplastic diseases is similar to that in the treatment of infectious diseases. However, there are important fundamental differences between the two. In microbial infections the offending invader 1) is "foreign" to the host 2) has biological requirements for growth and multiplication that

are sufficiently different from those of host cells to be susceptible to selective attack by chemical agents, 3) is vulnerable to efficient defense mechanisms of the host that supplement the action of the chemotherapeudic agent. On the other hand, the malignant cell 1) cannot be considered "foreign" and differs from the normal cell principally in respect to characteristics of relatively unrestrained growth as well as in aspects of differentiation 2) has biological characteristics that are qualitatively indistinguishable from those of a normal body cell 3) is so invulnerable to defense mechanisms of the host that presumable a single cell surviving chemotherapeudic attack would suffice to perpetuate the malignant process.

7. Models of Drug Action

As described in the previous section, many characteristics of drug action are complex and poorly understood. Mathematical models are devised in order to illuminate the problems involved. Two types of models dealing with drug action will be described briefly. The first type of model is devised to describe the transmission of the drug through a body, treating the localization of the drug at various sites, and the accumulation and elimination of drug from the system. These models are intended to apply to chemotherapy, but the emphasis is on drug distribution rather than on the details of action. A model of the second type treats the killing of the organisms, and is concerned with the success of treatment as measured by the number of individuals killed. In these

models, the problems of drug transport is recognized, but it plays a secondary role in the analysis.

a. Models of Drug Action--On An Individual.--The success of chemotherapy lies in being able to localize a drug selectively in a tumor. In the past such studies on chemotherapeutic agents were conducted by labeling them with radioactive isotopes. However, lacking a theoretical model, such studies have no productive properties. Much work has been done by Bellman, Jacquez, Kalaba (and Kotkin) (3,4,22) on models of the distribution of an injected drug within the body.

Their earlier work dealt with the processes occuring in the diffusion of a drug between capillary plasma, extracellular space and intracellular space. In the intracellular space the drug can react with certain components of the system. Equations were set up to describe the events, within a capillary bed. These events can be classified as 1) processes occuring in the peripheral capillary beds, and 2) situations arising in the circulation, i.e. in the connections between the capillary beds. Account must be taken of blood flow, capillary permeability, permeability of the cell membranes and the chemical interactions of the drug in intracellular space in order to construct a realistic model of the capillary bed. A model of circulation must include mixing, blood flow and time lags.

The authors set up a simplified model of the body to describe circulation of blood. They first divide the body into two compartments (organs), later into more (22). Each

organ is treated as a single capillary bed, linked to other organs via the circulation. Each organ is simulated by a black box with plasma containing the drug at some concentration entering on one side and leaving it at another. Differential equations describe the processes inside the box. Boundary conditions are imposed depending on the structural arrangements of the organs in relation to each other. The approach to this model is similar to that in engineering control theory. The resulting difference-differential equations for this model are complex and their solutions are constructed from results obtained from the analogous problems of engineering control.

Another class of studies of drug effects deals with the spacing of drug application to achieve a maximum therapeudic effect. Buell, et al. (6) base a model of optimal drug dosage regimens on the two compartment model of the body devised by Bellman, Jacquez and Kalaba. The criterion of success in this model is the spacing of treatment to achieve the plateau effect, i.e., maintainance of drug concentrations between prescribed lower and upper limits. The solution of a set of differential equations yields the initial dose, maintainance dose, and interval between doses.

b. Models of Action of Drug (Poison) which Affect the Growth of Populations.--Finney (13) describes the case of a single drug or poison applied to a population at various dose levels. From the number of survivors observed at each dose level , he estimates the mean and variance of the tol-

erance distribution of the population. Elegant iterative schemes (PROBIT ANALYSIS) are devised for the computations involved. He examines the treatment of complex organisms and he applies the results to cases of other quantal responses besides death. The usual assumption is made that an individual organism shows a quantal response to a drug if the dose exceeds its tolerance, a quantity characteristic of the individual at the time at which the drug acts (12,13). The tolerance for one drug varies from one individual to another in a population, and evidently a number of factors determine the individual tolerance. Presumably, for a response to occur, a certain minimum amount of drug (or active metabolite) must reach the site of action in the individual. This is always less than or equal to the quantity of drug applied, which quantity is normally used in computing the tolerance. However, for a correct mechanistic analysis of effectiveness, it is the quantity at the active site which is important (29). Tolerances for a single drug are very often log normally distributed (12, 13). Accordingly, the response in normal equivalent deviations is taken as linear in log dose.

The joint action of two drugs has been formulated by Bliss (5), and Finney (13) treated this subject in a more comprehensive manner. They set up biological models of joint action, and deduce mathematical models from them. The set of biological models, formulated for multicellular organisms, sprang from a two-way classification. The joint action was defined as similar or dissimilar according as the site of

primary action in the organism was the same or different, and as interactive or non-interactive according as one drug influenced or did not influence the biological action of the other. Hewlett and Plackett (19, 20, 29, 30, 31) attempt to fit these models to data. In general, the problem leads to equations which are awkward to use, but solutions to certain special cases of the models are found.

Plackett and Hewlett (29) suggest that next it would be interesting to introduce time dependent effects in the two drug problem, and to relate the effect to both the doses and the time of application. They suggest that the simplest cases of a scheme of this sort are likely to be those most usefully investigated such as "that of a single poison applied in more than one dose".

We followed this lead by first considering repeated drug application on more complex populations such as insects. Because of the availability of quantitative experiments on leukemic cells, and not on multicellular organisms, this study deals with unicellular organisms.

CHAPTER I

THE MODELS

I. Introduction

The analysis of the experiments is carried out by means of a simple mathematical model, or set of models. The model represents the growth of organisms during multiple application of a drug. The equations of the model must contain only a small number of parameters (see Chapter VI). As the need arises, additional parameters are considered in order to improve the fit of the model to a given set of experimental data. The fundamental properties of the system, expressed as parameters, are drug retention, cell growth and a distribution of sensitivities of the cells to the drug (tolerance distribution).

The practical objective is the analysis of drug treatments of L1210 leukemic cells in mice, and therefore certain facts, pertinent to this system are incorporated into the model. For example, it will be assumed that these cells do not grow for two days after intraperitoneal implantation in mice and that each cell dies immediately when the effective dose surpasses its tolerance to the drug (34). At first it is assumed that the growth rate is unaffected by the drug, although later this assumption is qualified in the case of one of the drugs (amethopterin).

The equations can be written for drug doses which are arbitrarily spaced and of unequal amount, but since the multiple dose experiments were performed using equally spaced, constant doses, only simplified formulae appropriate to this case are presented here. In addition to the models for growth by binary fission, another model is constructed in which the parent does not disappear in the reproductive process. An example of this is a population of insects in an agricultural experiment.

II. Properties of the Models

A. Underlying characteristics of all the models

Consider three principal aspects of the problem, the population growth, drug retention, and tolerance distribution.

1) Population Growth.--According to Skipper, et al., the growth pattern of leukemic cells in mice, apart from a two-day lag, is exponential and growth continues until death of the host intervenes. Since leukemic cells divide by binary fission, the increase in number is geometric. If one makes the seemingly reasonable assumption that host death is associated with the same critical number of leukemic cells, it becomes possible to calculate the basic constants from observations of the day of death of the host. It will be assumed in what is to follow that the lethal number of cells is 10^9 and that the time for cell division is 0.45 (see Chapter III, section II-B) days (34).

2) Drug Retention.--Evidence of drug retention is de-

monstrated in many laboratory experiments. The rate of drug excretion can be measured as well as the disappearance of drug from the circulatory system (36). The problem, however, is more complex. Some of the drug remains in the system and goes to what Veldstra (40) terms "sites of loss". Thus some portion of a dose of drug may remain stored in the tissues and some may be metabolized. When metabolized, a drug is usually changed into a less biologically active substance, but examples are known where the reverse occurs (19). It is reasonable to assume that the drug concentration in the host decays exponentially. This is approximately true for a number of drugs (36).

3) Log Normal Tolerance Distribution.--The assumption that the log of the tolerance is normally distributed has been treated by Finney (12, 13). This distribution is "robust" in the sense that small functional deviations should not have a major influence on the results.

B. Inheritance of tolerance to drug

Several possible relationships between tolerance of parent cells and of daughter cells can be constructed. Two extreme cases are considered here.

1) The tolerances of daughter cells resemble their parents'. In this case, no weaklings appear upon fission once weaker cells are already killed off. This model cannot possibly describe the leukemic cell data where many daily treatments are given. The multiple drug treatments would

have very little effect unless drug retention was very high. This is not compatible with cancer chemotherapy data.

2) Leukemic cells, appearing on fission, do not inherit the tolerance of the parent to the drug, but are characterized by the tolerance distribution of the original population. Accordingly, a treated population containing only cells in the upper portion of the tolerance distribution regeneratesa normal population upon cell division.

3) It is possible that these daughter cells may resemble the parents to a degree which could be specified with the addition of one or more parameters. This intermediate case could also have been considered. It was not tried here.

C. Other variations of the model

1) The growth rate of the cells might change with successive dosing. In fact, biochemical studies have shown that amethopterin reacts irreversibly with dihydrofolic reductase, an enzyme which plays an important role in nucleic acid replication (21). For this reason, the supposition that cell growth might change with drug dosage is reasonable, but by no means certain.

2) The mean of the tolerance distribution μ, might decrease with increased dosing, reflecting a weakened state of the leukemic cells.

3) It is not always true that a drug becomes effective immediately when applied. The "effective" drug concentration may be delayed depending on the series of chemical interactions which bring the drug to the site of action. This "time delay" could be incorporated into the model.

III. Description of the Models

Multiple drug treatments are applied to both simple unicellular species and more complicated organisms. In unicellular organisms, reproduction is by fission and the parent disappears on reproduction. For more advanced plants or animals, the parent remains after the offspring is born. The models are classified accordingly, and are called "Cell Model" and "Model With Surviving Parent" respectively.

Two extreme situations have been considered for cell models, CELL MODEL I where the tolerance of an offspring is unrelated to that of the parent, and CELL MODEL II, where the tolerance of the offspring is the same as that of the parent. CELL MODEL II does not apply to leukemia (see section II-B-1). A further modification of CELL MODEL I is examined, in which a population of leukemic cells appears which is resistant to the drug. This CELL MODEL WITH RESISTANT CELLS APPEARING will be designated as CELL MODEL IR.

A. Cell Model I

This model is based on the assumptions of population growth (II-A-1), drug retention (II-A-2), log normal tolerance distribution (II-A-3) as well as the assumption of

no inherited tolerance (II-B-2).[1]

1) Terminology

N_o = initial population

Z_o = initial dose

γ = time interval between treatments

N_k = population size after k^{th} treatment

Z_k = effective dose immediately after k^{th} treatment

q_k = fraction of current population killed by k^{th} dose

$p_k = 1-q_k$

α = growth rate of cells

β the exponential decay constant for retention of drug. The amount of drug retained at time t after injection of amount Z is $Ze^{-\beta t}$.

μ, σ^2 the mean and variance of original tolerance distribution

2) Mathematical Formulation

$$Z_k = Z_{k-1}e^{-\beta\gamma} + Z_o. \qquad k \geq 1$$

$$q_k = \int_{-\infty}^{\frac{\log_{10} Z_k - \mu}{\sigma}} \frac{e^{-\frac{1}{2}t^2}}{\sqrt{2\pi}}\, dt$$

$N_1 = N_o p_1$

$N_2 = N_o p_2$ (2 see footnote)

---

[1]Parenthesis refer to section headings in this chapter.

[2]For the first two days, the leukemia cells do not grow (34).

$$N_k = N_{k-1} p_k e^{d\gamma} \qquad k > 2$$

3) In Figure 1 a schematic plot of drug versus time during a multiple dose treatment is shown. In Figure 2 a typical plot of the number of cells present during the treatment is also shown as a function of time. In constructing this latter curve, it is assumed that the drug acts only at the moment of application. This assumption has been used throughout, and is admittedly a serious oversimplification. It introduces no complications when drug retention is short-lived, but in future work should be replaced by a more sophisticated treatment for those cases when the drug is retained in the animal for a long time.

FIGURE 1

DOSE vs. TIME

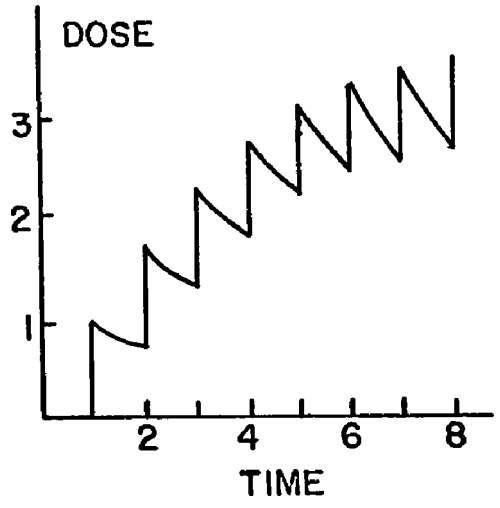
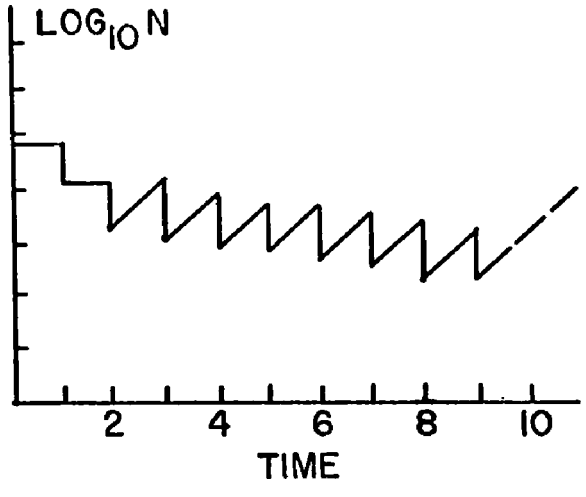


FIGURE 2

NUMBER OF SURVIVORS (N) vs. TIME



B. Cell Model II

This model is also based on the assumptions of population growth (II-A-1), drug retention (II-A-2), log normal

tolerance distribution (II-A-3), but differs from Cell Model I in that we assume that the tolerance of the daughter cells resulting from a cell division is the same as that of the parent cell (II-B-1). The terminology is the same as in Cell Model I. Modified equations are:

$$N_1 = N_o p_1$$

$$N_2 = N_o p_2$$

$$N_k = N_{k-1}\, e^{\alpha\gamma} \cdot \frac{p_k}{p_{k-1}} \qquad k > 2$$

This model, as previously stated (II-B-1) is not suitable for the leukemic cell data.

C. CELL MODEL IR (Cell Model with Resistant Cells Appearing)

In addition to the standard assumptions of drug retention (II-A-1), exponential growth (II-A-2), log normal tolerance distribution (II-A-3), and resemblance of daughter cells to original population (II-B-2), i.e., Cell Model I, we introduce the hypothesis of a developing drug resistant strain. These cells are generated on reproduction at a small constant rate, $p \sim 10^{-5}$. The mean and variance of the resistant strain is taken to be of such size that no cells are killed by the applied drug. Therefore, the only significant additional parameter is the generation rate of the resistant strain.

1) Further assumptions for this model

a) Before treatment, there are no resistant cells. This is not accurate, but in a normal population the fraction

of resistant cells is so small that their neglect is numerically trivial.

b) Resistant cells generate only resistant cells.

c) The growth rates of resistant and normal cells are identical.

2) Some additional terminology

p = fraction of normal cells which become resistant per day.

q_k^* = fraction of resistant population killed by k^{th} dose, ≈ 0.

N_k^n = normal population size immediately after k treatments.

N_k^r = resistant population size immediately after k treatments.

3) Mathematical formulation

$$Z_k = Z_{k-1}\, e^{-\beta\gamma} + Z_o \qquad k \geq 1$$

$$q_k = \int_{-\infty}^{\frac{\log_{10} Z_k - \mu}{\sigma}} \frac{e^{-\frac{1}{2}t^2}}{\sqrt{2\pi}}\, dt$$

$$q_k^* = \int_{-\infty}^{\frac{\log_{10} Z_k - \mu_r}{\sigma_r}} \frac{e^{-\frac{1}{2}t^2}}{\sqrt{2\pi}}\, dt \quad (^1\text{see footnote})$$

$$p_k^* = 1 - q_k^*$$

---

[1] μ_r is so large that $q_k^* \approx 0$.

$$N_1^n = N_o p_1$$

$$N_1^r = N_2^r = 0$$

$$N_2^n = N_o p_1 p_2$$

$$N_k^n = N_{k-1}^n \, p_k (1-P) \, e^{\alpha\gamma} \qquad k > 2$$

$$N_k^r = (N_{k-1}^r + P \, N_{k-1}^n) \, e^{\alpha\gamma} \cdot p_k^* \qquad k > 2$$

$$N_k = N_k^r + N_k^n$$

D. <u>The Model with Surviving Parent</u>

While the previous models are devised for bacteria or cell populations, it is evident that models of a similar form might be applicable to control of insects in agriculture, for example, where the birth of new organisms does not imply the disappearance of the parent. The equations are somewhat more complex than those given above, but the spirit of the analysis is the same, and computations are no more difficult. Much of the preliminary work on choosing criteria for best fit and techniques of parameter estimation was done on this model. The assumption for this model are identical to Cell Model I (drug retention (II-A-2), exponential growth (II-A-1), log normal distribution (II-A-3), resemblance of daughter cells to original population (II-B-2)), the difference being that the parent does not disappear at birth of offspring (III). The terminology is also identical to Cell Model I.

Mathematical Formulation Using Same Terminology as in Cell Model I

$$N_1 = N_o p_1$$

$$N_2 = N_o p_2$$

$$N_3 = N_o p_3 + N_o p_2 p_3 (e^{\alpha\gamma} - 1) = N_o p_3 (q_2 + p_2 e^{\alpha\gamma})$$

$$N_k = N_{k-1} (q_{k-1} + p_{k-1} e^{\alpha\gamma}) \frac{p_k}{p_{k-1}} \cdot \qquad k \geq 3$$

IV. Summary

These models have been developed as starting points for the analysis of multiple drug treatments. Cell Models I and IR were applied to one set of experiments in which L1210 leukemia in mice was treated with amethopterin, and to two sets of data using NSC 409962. The Model with Surviving Parent was studied with artificial data obtained with use of a random number generator. Techniques of minimization and trials of various criteria for "best fit" were explored with this model. The Model with Surviving Parent is not suitable to the leukemia problem where reproduction of cells is by binary fission. It was also found that Cell Model II, in which the offspring has the same tolerance to the drug as the parent, was not at all consistent with the facts of L1210 leukemic cell growth (II-B-1).

CHAPTER II

STATISTICAL ANALYSIS

I. Introduction

The success of a model depends on its suitability for analysis of experiments. This success depends on being able to find a set of "best" parameters, incorporating these, and then observing how well the data fits the model. The criterion of "fit" is not obvious; a least squares method was used. The way a least squares procedure is formulated is not unique, and the choice made in this study is discussed in this chapter.

The problem of searching out the "best" parameters is not elementary, the functions treated are highly non-linear, and contain three or more parameters. The nature of the problem is that ordinary procedures of minimization do not guarantee that a minimum can be found. Many techniques were tried. These will also be discussed.

II. Criteria for Best Fit

In the first calculations, parameters were estimated using a least squares criterion based on the number of surviving cells immediately after the last (k^{th}) treatment in each of n experiments, (N_n^k). This led to the minimization of the function:

$$Q_1 = \sum_n \left[N_n^k \text{ (obs.)} - N_n^k \text{ (calc.)}\right]^2$$

This had an obvious disadvantage. The N_n^k (calc.) values for experiments with high drug doses and many treatments were small numbers, while low drug doses led to large N_n^k (calc.). Obviously, the statistical weights of the large numbers were out of proportion for a reasonable evaluation.

Another criterion then tried was

$$Q_2 = \sum_n \frac{\left[(N_n^k \text{ (obs.)} - N_n^k \text{ (calc)}\right]^2}{N_n^k \text{ (calc.)}}$$

which gave relatively equal weights to small and large N_n^k (calc.). It suffered, however, from the fact that a simple physical meaning could not be attached to Q_2.

This disadvantage was removed in the criterion

$$Q_3 = \sum_n \frac{\left[N_n^k \text{ (obs.)} - N_n^k \text{ (calc.)}\right]^2}{N_n^k \text{ (calc.)}^2}$$

a function which reduced the statistical weights for large N_n^k (calc.) even further. Here, a fairly simple justification exists. Define a number R_n, for each of the n experiments

$$R_n = \frac{\text{obs. no. of survivors}}{\text{calc. no. of survivors}} .$$

Then

$$Q_3 = \sum_n (R_n - 1)^2 .$$

Since $R_n = 1$ represents ideal fit of the model, Q_3 seems to be a better criterion function than Q_1 or Q_2.

These three criteria all had an additional common deficiency. They were all based on survivors at the last treatment, no matter how many treatments were used in each experiment. Some experiments have just a few treatments, others have many. This unbalanced the statistical weights of the experiments. This is corrected by a calculation on the day of death, which automatically weights all experiments equally, since on that day, the observed number of cells is taken as a uniform 10^9. Thus we arrive at

$$Q_4 = \sum_n (10^9 - \tilde{N}_n)^2,$$

or at

$$Q_4^* = \sum_n \frac{(10^9 - \tilde{N}_n)^2}{(\tilde{N}_n)^2}$$

where $\tilde{N}_n$ is the calculated number of survivors on day of death, in the n^{th} experiment, regardless of how many treatments were given.

Both Q_4 and Q_4^* are still unsatisfactory. This can be shown in the following way. Imagine two experiments where N_n takes on the values 10^8 and 10^{10}, respectively. Since growth is exponential, our subjective feeling is that the contribution to the least squares function should be the same for each result. Neither Q_4 nor Q_4^* possess this characteristic.

Since the growth process is essentially exponential, a least squares criterion based on the logarithms of the number of cells was formulated.

This is

$$Q_5 = \sum_n (\log 10^9 - \log \tilde{N}_n)^2 = \sum_n \log^2 \frac{10^9}{\tilde{N}_n} .$$

This treats errors with a factor of 10 too high or too low equally, does not give undue statistical weight to high or low numbers, and is a natural form in a system where geometric growth is predominant. A physical explanation for the use of Q_5 is provided in the fact that

$$Q_5 = C\, Q_6$$

where

$$Q_6 = \sum_n (\text{day of death (obs.)} - \text{day of death (calc.)})^2$$

where

$$C = (.43429\alpha)^2$$

(Appendix A). It is apparent that minimization of either Q_5 or Q_6 leads to the same values of the parameters.

III. Minimization

A. Discussion of the Problem

The parameters in the model are to be obtained by minimization of Q_5. Analytically, the minimum is found, in principle, by setting the derivative of Q_5 with respect

to each parameter equal to zero, solving the resultant non-linear equations for all solutions, and selecting the smallest Q among the extrema so found. However in practice this is hard to accomplish, if not impossible, and other techniques must be used to find the minimum Q_5.

Numerically, this is done by picking a starting point in the parameter space and searching for a minimum Q_5 in the direction of the gradient $Q(\underline{\theta})$, where $\underline{\theta}$ is the parameter vector (β, μ, σ^2...). Since multiple minima often exist, this procedure may lead to a trivial local minimum, or a minimum at a point $\underline{\theta}$ outside the physical range of the parameters (for example, a negative variance). This type of error can often be avoided by choosing many different starting points for this calculation or by first fixing one or more parameters and scanning the reduced sample space for minima.

It should be emphasized that there is no mathematical certainty that the best fit will be found with highly non-linear equations. The difficulties increase enormously with each new parameter. A good rule for a successful minimization is to have just a very few parameters and, if possible, to estimate some of these independently from outside information.

B. Various Methods for Estimating Parameters of A Nonlinear System

1) The linearization (Taylor Series) method

N_k ($\underline{\theta}$), the observed number of survivors on the day of death, is expanded in a Taylor Series about a point

$\underline{\theta}_0 = (\beta_0, \mu_0, \sigma_0^2 \ldots)$, obtained as a first estimate, ignoring terms of power higher than one. This leads to n linear equations (n - no. of experiments) in the variables $(\Delta\beta, \Delta\mu, \Delta\sigma^2 \ldots)$ which are solved by the use of linear regression. If the increments, $\Delta\beta$, $\Delta\mu$, $\Delta\sigma^2$, are not small enough, the procedure is repeated, expanding about $\beta_1 = \beta_0 + \Delta\beta$, $\mu_1 = \mu_0 + \Delta\mu$, $\sigma_1^2 = \sigma_0^2 + \Delta\sigma^2$. This is iterated until the parameters no longer change. A "Scientific Subroutine Package" called DLLSQ was used for the linear regression calculations on an IBM360 computer. Additional disadvantages are:

a) Convergence may be slow, requiring many iterations.

b) Initially, the function Q may oscillate wildly, continually reversing direction. Nevertheless, the solutions may stabilize eventualy.

c) The method may not converge at all, increasing the sums of squares at each iteration. For further discussion see reference (11).

2) Gradient Methods

An iterative process is used to search for the minimum of Q. The basic idea is to move from an initial point ($\underline{\theta}_0$) along the gradient vector in specified step sizes. It is easy enough, for any of the models, here described, to find analytic expressions for the partial derivatives of Q with respect to each parameter.

Two computer programs provided by "Scientific Subroutine Package" were used in an IBM360 computer. They are

1. Fletcher Powell Subroutine DFMFP (14).
2. Conjugate Gradient Subroutine DFMCG (15).

Both programs are of equal applicability. No effort was made to improve on these computer programs, with the exception of scale variations.

Disadvantages - While, theoretically, these methods will converge, they may do so with agonizing slowness after some initial progress. This is specially true for the Q value used here, where many contours are "banana-shaped". In such cases, the path along the gradient zigzags slowly across a narrow ridge, each iteration bringing only slight reduction in Q and minute changes in the parameters.

3) Direct Search Method

From the theoretical point of view this is the simplest technique. For the functions appearing in these models, it works well and converges quickly. (See Appendix B.) It has the added advantage of being useable on an IBM1130 computer, as well as the more expensive IBM360. A point is chosen and initial increments, both positive and negative, are supplied for each parameter. A grid of 2^k points is obtained, k being the number of parameters being varied.

Q is evaluated at each point of this grid. The point providing the lowest value of Q is the next starting point in the search for a minimum. If no improved point is

found, the increments are reduced, and, again, a search of the resulting grid effected. The original computer program was provided by Professor Brice Carnahan and adapted to this problem.

C. The Importance of A Good Starting Point

All iterative procedures described in Section III-B require arbitrary initial values of the parameters. Unless the starting point is sufficiently close[1], convergence to the minimum is very slow, or if there are several local minima, convergence may be to an unwanted local minimum. It is often apparent when a minimum point does not correspond to the desired result, sometimes because the parameter values are physically unrealistic, and sometimes because lower values of Q are found in a different region of parameter space. See Appendix B.

D. Discussion

In order to study and compare the techniques discussed in Section III-B, a simulated set of data was used. It was theorized that a population of 100 (N_0), is subjected to k = 10 treatments of dose $Z_0 = 1$, spaced $\gamma = 1$ days apart. Starting with a hypothetical set of parameters ($\alpha = .4$, $\beta = .8$, $\mu = .47712$, $\sigma^2 = 1$), and using the Model with Surviving Parent, the survivors after each treatment were generated

[1]The term "sufficiently close" is not well defined except in terms of subsequent operations. The change in the function with respect to the parameters, and the existence of extraneous extrema are the controlling factors.

by Monte Carlo techniques (68, 83, 105, 134, 172, 221, 285, 367, 473, 609).

Both the initial values and initial step sizes in the iteration process were varied and their effect on convergence noted. The numerical results will be summarized in Appendix B. After extensive experimentation with minimization techniques and with varying numbers of unknown parameters, it was decided to 1) reduce the number of parameters by estimating α independently, and, 2) use the Direct Search method on real data because it was the most convenient.

CHAPTER III

RESULTS OF DETERMINATION OF PARAMETERS

I. Introduction

Data obtained by Skipper, et al., (34) on the effects of multiple dose treatments of L1210 leukemia in mice using two anticancer drugs amethopterin (Methotrexate) and NSC 409962 (1, 3 - bis - (2 chloroethyl) - 1 - nitrosourea) were studied using the models discussed in Chapter I. In all the experiments 10^5 leukemic cells were injected intraperitoneally into groups of 10 mice (some experiments on only 6 mice). These cells did not begin proliferating until two days later. One day after transplantation treatment was begun. This consisted of equal doses of drug given at one day intervals. Both the dose and length of treatment were varied and the average day of death recorded.

The parameters were determined by minimization of the least squares function Q_5 (Chapter II, Section II). The Direct Search method of Carnahan (Chapter II, Section III-B-3) was used.

II. NSC 409962

A. Introduction

This drug belongs to the class of nitrogen mustards. Between World Wars I and II extensive studies were made of the

biological and chemical action of these compounds, which had been previously used only in chemical warfare. It was noted that they readily gain access to the circulatory system, cause profound local injury, serious systematic intoxication, and dissolution of lymphoid tissue. This marked cytotoxic action on lymphoid tissue led to the study of the effect of nitrogen mustards on transplanted lymphosarcoma in mice. It was demonstrated that extensive regression of established tumors could be produced by doses of nitrogen mustards that were tolerated by the host.

Nitrogen mustards are chemically reactive alkylating agents. It is postulated that their cytotoxic effect derives from their ability to crosslink DNA, and to form strong bonds between DNA and protein as well as between two protein molecules. They thus disturb the fundamental mechanisms concerned with cell growth and function. The ability of these drugs to interfere with normal mitosis in all rapidly proliferating tissues provides the basis for their therapeutic properties. There is a great difference in the susceptibility of various cell types to this effect of alkylating agents, and, in fact, lymphocytes are very sensitive in contrast to most other cells (17).

Two different sets of data were analyzed. Both Cell Model I and the Cell Model IR (Chapter I, Section II) were applied and comparisons made. Possible toxicity of high dosages of drug to the host was also investigated.

B. Data Set I

The data is presented in Table 1 (p.47). It should be noted that only three dose levels are used (8, 5.4, 3.6 mg/kg.wt.) in these 27 experiments. The growth rate α was taken as 1.53506, corresponding to an eight day life span of control mice, assuming that cell division begins two days after injection of cells (doubling time equals 0.45 days). In Cell Model IR, the mean and variance of the tolerance distribution of the resistant strain were chosen so that hardly any resistant cells were being killed (i.e., $\mu_r = 7$, $\sigma_r^2 = 1$). The results of the parameter estimation are given in Table 2 for both models studied.

TABLE 2

PARAMETERS OBTAINED FOR NSC 409962 - DATA SET I BY MINIMIZATION

Values	Cell Model I	Cell Model IR
μ	.425125	.431425
σ^2	.09988	.053364
β	$\approx\infty$ *	$\approx\infty$ *
p	0.0	$.1381 \times 10^{-5}$
Q_5	42.05	25.1

* For computer calculation, β was taken to be 6.1

As mentioned in Chapter II, there were three varying parameters in Cell Model I, β, the decay constant, as well as μ and σ^2, the mean and variance, of the tolerance

TABLE 1

DATA SET I - NSC 409962

Experiment Number	Dose in mg/kg.wt.	Number of Treatments	Average Day of Death
1	8.0	1	12.5
2	8.0	2	14
3	8.0	3	16
4	8.0	4	19
5	8.0	5	20
6	8.0	7	20
7	8.0	9	21.5
8	5.4	1	9.5
9	5.4	2	11
10	5.4	3	13
11	5.4	4	14
12	5.4	5	16
13	5.4	7	17.5
14	5.4	9	16.5
15	5.4	11	19
16	5.4	13	21.5
17	5.4	15	24
18	3.6	1	9
19	3.6	2	11
20	3.6	3	11.5
21	3.6	4	12
22	3.6	5	13
23	3.6	7	13.5
24	3.6	9	14.5
25	3.6	11	16
26	3.6	13	18
27	3.6	15	18.5

distribution. In the Cell Model IR, there is an additional free parameter P, the fraction of normal cells becoming resistant each day. It should be noted that Q_5 has been substantially reduced in the second model. The mean μ is fairly stable, and the variance in the second model is lower.

By taking the parameters listed in Table 2, the calculated number of cells at the day of death (N_k (calc.)) were compared with the observed number 10^9. This is expressed for each experiment as $R_k = 10^9/N_k$ (calc.), and measures how well the fitted model fits the data. These are listed in Tables 3 and 4. Figures 3 and 4 contain the same information in graphical form.

C. Data Set II - NSC 409962

The data for this set of experiments is presented in Table 5. This set is based on experiments using many different doses, in contrast with the experiments in Data Set I. The fluctuations in the experimental data becomes evident from observation of replicated treatment. For example, using a dose of 8 mg/kg.wt. and 15 treatments, the recorded average day of death varies from 26.5 to 21.5. This is not surprising when dealing with biological materials, but could be indicative of a varying degree of toxicity to the host at higher dosages.

As in Data Set I, α, the growth rate is taken as 1.53506. In Table 6, the parameters μ, σ^2 and β obtained at a minimum Q_5, and using Cell Model I, are given.

TABLE 3

VALUES OF N_k, R_k, LOG R_k, CALCULATED MINUS OBSERVED DAY OF DEATH FOR NSC 409962 EXPERIMENTS OF DATA SET I, CELL MODEL I, USING PARAMETERS LISTED IN TABLE 2

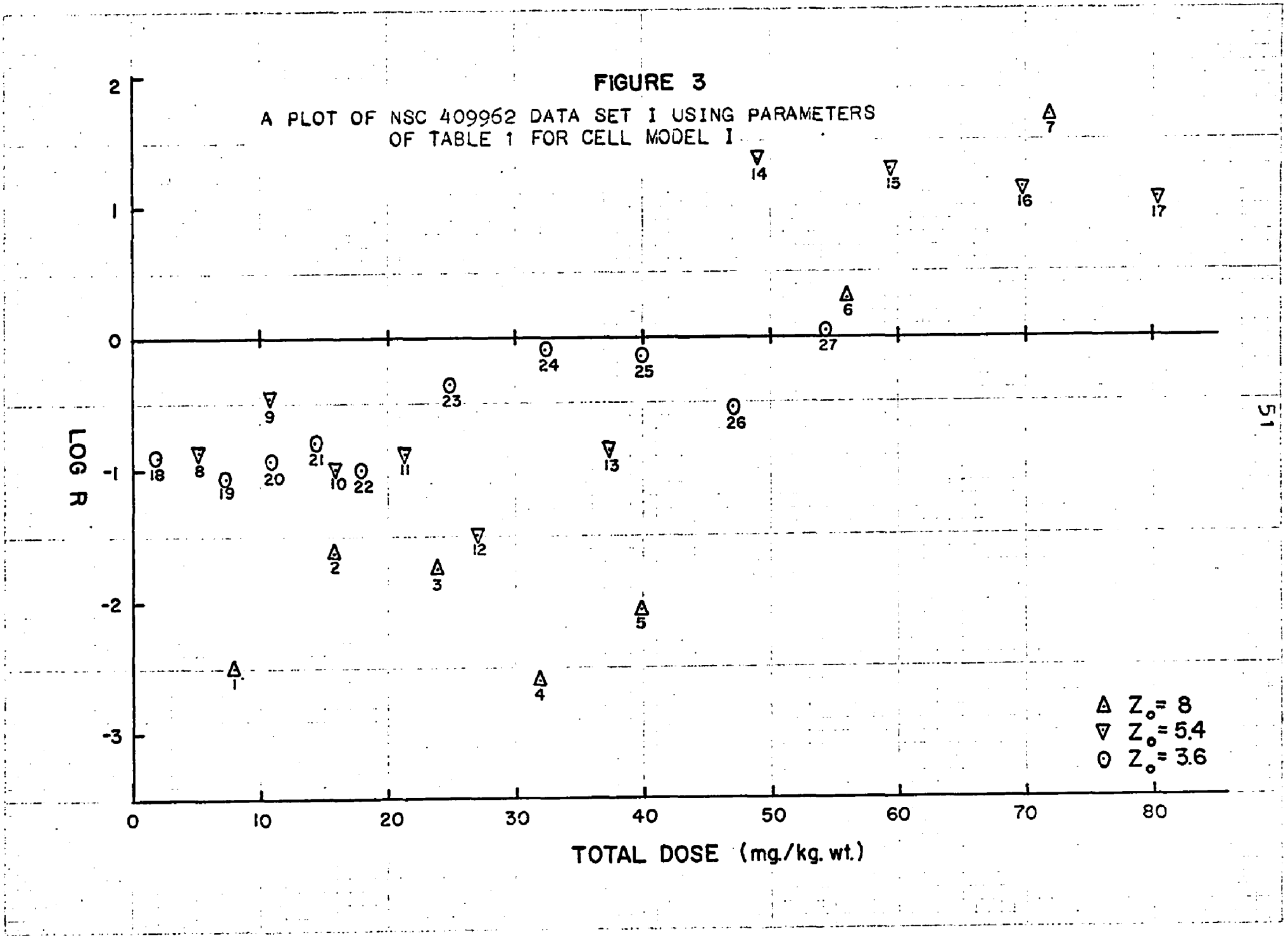
Experiment Number (k)	N_k	R_k	$\text{Log}_{10} R_k$	Calculated-Observed* Day of Death
1	6,513.8	.003	-2.480	-3.72
2	421.7	.023	-1.625	-2.44
3	126.7	.016	-1.770	-2.65
4	38.0	.002	-2.581	-3.87
5	11.4	.008	-2.059	-3.09
6	1.0	2.084	.319	.48
7	0.0	49.716	1.696	2.54
8	16,568.0	.130	- .886	-1.33
9	2,732.3	.365	- .437	- .65
10	2,091.5	.103	- .987	-1.48
11	1,601.0	.134	- .871	-1.31
12	1,225.5	.037	-1.422	-2.13
13	718.1	.139	- .856	-1.28
14	420.7	23.764	1.376	2.06
15	246.5	18.824	1.275	1.91
16	144.4	14.912	1.174	1.76
17	84.6	11.812	1.072	1.61
18	33,884.8	.136	- .863	-1.30
19	11,443.6	.087	-1.059	-1.59
20	17,938.4	.120	- .920	-1.38
21	28,119.5	.165	- .782	-1.17
22	44,078.8	.105	- .978	-1.47
23	108,311.5	.428	- .368	- .55
24	266,145.6	.809	- .092	- .14
25	653,979.1	.709	- .149	- .22
26	1,606,972.5	.288	- .539	- .81
27	3,948,689.7	1.175	.070	.11

* Column 4 $= \dfrac{\log_e R}{\alpha}$

TABLE 4

VALUES OF N_k, R_k, LOG R_k, CALCULATED MINUS OBSERVED DAY OF DEATH FOR NSC 409962 EXPERIMENTS OF DATA SET I, CELL MODEL IR, USING PARAMETERS LISTED IN TABLE 2

Experiment Number (k)	N_k	R_k	Log R_k	Calculated-Observed Day of Death
1	2,042.3	.010	-1.977	-2.97
2	41.3	.242	- .616	- .92
3	3.9	.556	- .255	- .38
4	.4	.274	- .562	- .84
5	0.0	2.504	.399	.60
6	.1	17.141	1.234	1.85
7	2.7	1.718	.235	.35
8	9,642.8	.223	- .651	- .98
9	922.9	1.084	.035	.05
10	410.0	.525	- .279	- .42
11	182.1	1.183	.73	.11
12	81.0	.573	- .242	- .36
13	19.0	5.263	.721	1.08
14	68.6	145.834	2.164	3.25
15	1,410.0	3.292	.517	.78
16	30,365.4	.071	-1.149	-1.72
17	654,203.1	.002	-2.816	-4.22
18	29,418.6	.158	- .802	-1.20
19	8,612.0	.116	- .935	-1.40
20	11,701.6	.184	- .735	-1.10
21	15,899.9	.292	- .535	- .80
22	21,605.4	.215	- .668	-1.00
23	39,921.4	1.163	.065	.10
24	74,417.6	2.895	.462	.69
25	152,766.5	3.038	.483	.72
26	613,289.2	.757	- .121	- .18
27	8,268,835.7	.561	- .251	- .38


FIGURE 3
A PLOT OF NSC 409962 DATA SET I USING PARAMETERS OF TABLE 1 FOR CELL MODEL I
LOG R
TOTAL DOSE (mg./kg. wt.)
2
1
0
-1
-2
-3
0
10
20
30
40
50
60
70
80
Δ Z₀ = 8
∇ Z₀ = 5.4
⊙ Z₀ = 3.6
51

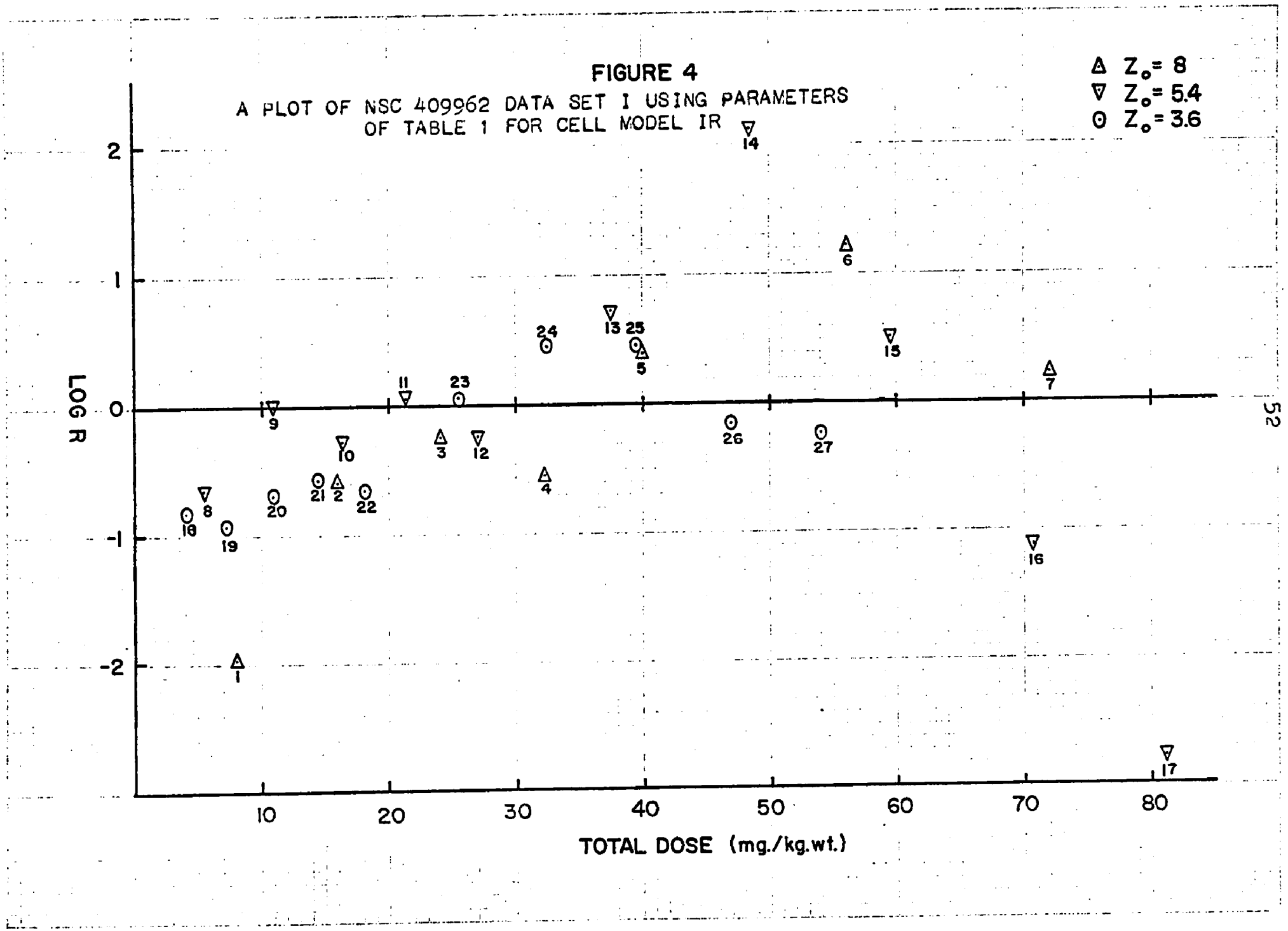

FIGURE 4
A PLOT OF NSC 409962 DATA SET I USING PARAMETERS OF TABLE 1 FOR CELL MODEL IR
Δ Z_o= 8
▽ Z_o= 5.4
⊙ Z_o= 3.6
LOG R
TOTAL DOSE (mg./kg.wt.)
2
1
0
-1
-2
10
20
30
40
50
60
70
80
52

TABLE 5

DATA SET II - NSC 409962

Experiment Number	Dose in mg/kg.wt.	Number of Treatments	Average Day of Death
1	8.0	15	26.5
2	6.6	15	23
3	4.4	15	21
4	3.0	13	14
5	10.0	15	32
6	8.0	15	25.5
7	6.6	15	23.5
8	4.4	15	21.5
9	3.0	12	13
10	2.0	11	12
11	9.0	15	25
12	6.0	15	20
13	4.0	15	18
14	2.6	12	13.5
15	10.0	15	29
16	8.0	15	21.5
17	6.6	15	19.5
18	3.0	12	13
19	2.0	12	13
20	6.3	24	25.5
21	3.12	18	19
22	1.56	10	10.5
23	.73	8	8.5
24	6.3	20	21
25	4.1	14	15
26	2.75	14	14.5

TABLE 6

PARAMETERS OBTAINED FOR THE MINIMUM POINT AND AT A NEIGHBORING POINT
NSC 409962 - DATA SET II - CELL MODEL I

Values	Minimum Point	Neighboring Point*
μ	.461679	.508249
σ^2	.249911	.247499
β	$\approx\infty$ *	2.24125
Q_5	57.41	57.73

*For subsequent calculation, β was set equal to 6.

TABLE 7

PARAMETERS OBTAINED FOR THE MINIMUM POINT AND AT A NEIGHBORING POINT
NSC 409962 - DATA SET II - CELL MODEL IR

Values	Minimum Point	Neighboring Point*
μ	.5076	.459602
σ^2	.02049	.02411
β	2.05287	4.
P	$.1342\ 10^{-5}$	10^{-6}
Q_5	57.07	57.25

*See text for description of "neighboring point"

In Table 7, the assumption of a developing resistant cell strain is introduced (Cell Model IR), and new parameter values at the minimum are obtained. The numerical values of the mean, μ, are different. This is not entirely due to the change in the model, but also reflects the fact that these minima are very flat. It is possible to change the parameters and still find a least squares function, Q_5, which is very close to the value at the minimum. For example, in Table 6 and 7 two sets of parameters are presented which are referred to as "neighboring points". These points were obtained in the course of calculations leading to minima, and are presented here to illustrate that small variations in Q_5 are consistent with substantial changes in the parameters. In this case, almost constant values of Q_5 are obtained by increases in β and compensatory decreases of μ.

The particular values of the parameters chosen for the "neighboring points" were selected from a large collection of available points to demonstrate that the μ value at the "neighboring point" in Table 7 is close to the μ value at the minimum point in Table 6 and vice versa. Accordingly, the different values of β obtained at the minima in the models with and without the assumption of drug resistance are not significant.

Tables 8 and 9 contain the computed values of R_k ($R_k = 10^9/N_k$ (calc.)). Figure 5 shows log R versus total dose for the experiments analyzed with and without the assumption of the appearance of a resistant strain of cells.

TABLE 8

VALUES OF N_k, R_k, LOG R_k, CALCULATED MINUS OBSERVED DAY OF DEATH FOR NSC 409962 FOR DATA SET II, CELL MODEL I, USING PARAMETERS AT MINIMUM POINT LISTED IN TABLE 6

Experiment Number (k)	N_k	R_k	Log R_k	Calculated-Observed Day of Death
1	615.6	.034	-1.456	-2.18
2	18,805.7	.246	- .608	- .91
3	9,232,046.2	.010	-1.965	-2.95
4	189,439,377.7	1.137	.056	.08
5	7.6	.000	-3.219	-4.83
6	615.6	.162	- .789	-1.18
7	18,805.7	.114	- .941	-1.41
8	9,232,046.2	.005	-2.299	-3.45
9	83,797,997.4	2.570	.410	.62
10	579,482,750.5	.371	- .430	- .64
11	64.4	3.341	.524	.79
12	90,991.3	5.101	.708	1.06
13	32,774,832.6	.305	- .516	- .77
14	265,793,461.8	.376	- .425	- .64
15	7.6	.060	-1.219	-1.83
16	615.6	75.391	1.877	2.82
17	18,805.7	53.174	1.726	2.59
18	83,797,997.4	2.570	.410	.62
19	1,682,508,474.7	.128	- .893	-1.34
20	149,553.2	668.654	2.825	4.24
21	6,742,766,580.2	.031	-1.495	-2.24
22	651,232,207.2	.712	- .147	- .22
23	374,330,296.9	1.239	.093	.14
24	84,127.3	2,560.911	3.408	5.11
25	13,462,985.1	16.002	1.204	1.81
26	985,000,690.4	.471	- .325	- .49

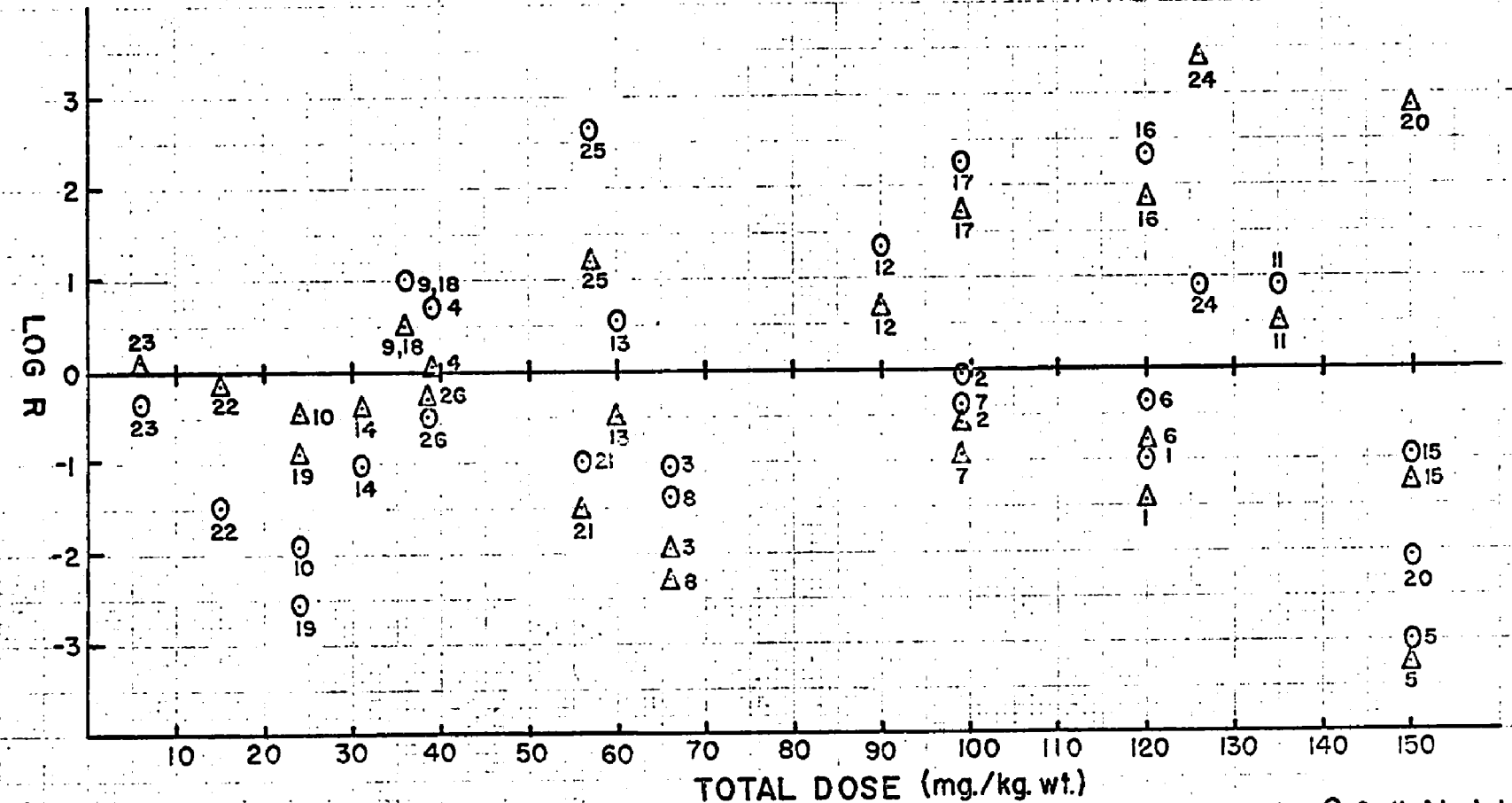
TABLE 9

VALUES OF N_k, R_k, LOG R_k, CALCULATED MINUS OBSERVED DAY OF DEATH FOR NSC 409962 FOR DATA SET II, CELL MODEL IR, USING PARAMETERS AT MINIMUM POINT LISTED IN TABLE 7

Experiment Number (k)	N_k	R_k	Log R_k	Calculated-Observed Day of Death
1	209.4	.103	- .988	-1.48
2	5,109.3	.908	- .042	- .06
3	1,102,323.5	.091	-1.042	-1.56
4	40,704,954.4	5.293	.724	1.09
5	4.3	.001	-2.966	-4.45
6	209.4	.477	- .321	- .48
7	5,109.3	.422	- .375	- .56
8	1,102,323.5	.042	-1.376	-2.06
9	20,523,730.4	10.497	1.021	1.53
10	17,977,330,407.7	.012	-1.921	-2.88
11	27.1	7.951	.900	1.35
12	21,996.0	21.102	1.324	1.99
13	2,819,371.8	3.547	.550	.82
14	1,101,652,801.5	.091	-1.042	-1.56
15	4.3	.108	- .966	-1.45
16	209.4	221.626	2.346	3.52
17	5,109.3	195.718	2.292	3.44
18	20,523,730.4	10.497	1.021	1.53
19	70,720,644,335.9	.003	-2.516	-3.77
20	10,553,140,805.1	.009	-2.023	-3.04
21	2,241,693,729.4	.096	-1.017	-1.53
22	15,168,548,144.5	.031	-1.514	-2.27
23	999,517,306.9	.464	- .333	- .50
24	22,735,787.6	9.476	.977	1.46
25	481,279.6	447.646	2.651	3.98
26	1,610,320,956.8	.288	- .540	- .81

FIGURE 5

A PLOT OF NSC 409962 DATA SET II USING RESULTS OF TABLES 8 AND 9



Since large total doses of drug are known to have a toxic effect on the host (34) of Data Set II (total cumulative doses less than 88 mg/kg.wt.) were analyzed separately. This shall be designated as Data Set II*. This truncated set is reproduced in Table 10 (p. 60).

The results of finding the parameters for minimum Q_5 using Cell Model I are presented in Table 11. The unusually low β value at the minimum has less significance than would be anticipated. This will be treated in Chapter IV. Cell Model IR was not studied on Data Set II* because it was not suitable for the detection of a developing resistant strain. See discussion Chapter IV.

TABLE 11

PARAMETERS OBTAINED FOR THE MINIMUM POINT AND AT A NEIGHBORING NSC 409962 - DATA SET II* - CELL MODEL I

Values	Minimum Point	Neighboring Point
μ	1.16809	.430078
σ^2	.246835	.189521
β	.0578125	4.
Q_5	8.07403	9.880752

Tables 12 and 13 contain calculations of N_k, R_k, log R_k, calculated minus observed day of death, for NSC 409962 using the parameters at the minimum point and at the neighboring point (Table 11). The neighboring point here is chosen for the purpose of comparing the mean μ with the mean at the

TABLE 10

DATA SET II* - NSC 409962 - CONSISTING OF ALL EXPERIMENTS IN DATA SET II WHOSE TOTAL DOSE IS LESS THAN 88 mg/kg.wt.

Experiment Number	Dose in mg/kg.wt.	Number of Treatments	Average Day of Death
1	4.4	15	21
2	3.0	13	14
3	4.4	15	21.5
4	3.0	12	13
5	2.0	11	12
6	4.0	15	18
7	2.6	12	13.5
8	2.0	12	13
9	3.12	18	19
10	1.56	10	10.5
11	0.73	8	8.5
12	4.1	14	15
13	2.75	14	14.5
14	3.0	12	13

TABLE 12

VALUES OF N_k, R_k, LOG R_k, CALCULATED MINUS OBSERVED DAY OF DEATH FOR NSC 409962, DATA SET II*, CELL MODEL I, USING PARAMETERS OF MINIMUM POINT LISTED IN TABLE 11

Experiment Number (k)	N_k	R_k	Log R_k	Calculated-Observed Day of Death
1	1,029,397.6	.097	-1.013	-1.52
2	97,828,030.8	2.202	.343	.51
3	1,029,397.6	.045	-1.346	-2.02
4	74,041,616.5	2.909	.464	.70
5	693,511,555.2	.310	- .508	- .76
6	3,927,797.1	2.545	.406	.61
7	230,558,980.1	.433	- .363	- .54
8	1,397,488,828.6	.154	- .812	-1.22
9	108,237,092.0	1.990	.299	.45
10	931,910,716.2	.498	- .303	- .45
11	497,755,474.4	.932	- .030	- .05
12	3,379,338.3	63.752	1.804	2.71
13	296,887,790.1	1.563	.194	.29
14	74,041,616.5	2.909	.464	.70

TABLE 13

VALUES OF N_k, R_k, LOG R_k, CALCULATED MINUS OBSERVED DAY OF DEATH FOR NSC 409962, DATA SET II*, CELL MODEL I, USING PARAMETERS OF NEIGHBORING POINT LISTED IN TABLE 11

Experiment Number (k)	N_k	R_k	Log R_k	Calculated-Observed Day of Death
1	884,891.7	.113	- .947	-1.42
2	67,756,703.5	3.179	.502	.75
3	884,891.7	.052	-1.280	-1.92
4	32,446,745.7	6.639	.822	1.23
5	436,435,940.7	.493	- .307	- .46
6	4,330,940.8	2.308	.363	.55
7	133,985,784.4	.746	- .127	- .19
8	1,234,722,718.4	.174	- .758	-1.14
9	1,433,201,198.5	.150	- .823	-1.23
10	620,689,277.2	.747	- .126	- .19
11	432,808,168.9	1.072	.030	.05
12	1,888,357.9	114.090	2.057	3.09
13	396,783,793.0	1.169	.068	.10
14	32,446,745.7	6.639	.822	1.23

"neighboring" point in Table 7 by choosing the same β values for the two points. (See earlier discussion of "neighboring point" in this section.) The data of Table 12 is shown in Figure 6 (log R versus total dose) together with the high dose experiments of Data Set II using the parameters of the minimum point of Table 11. In Figure 7, a similar plot is shown using the parameters of the neighboring point in Table 11. The data in Figure 6 and 7 is also shown in Table 14 and 15.

Note that most of the high total-dose points in Tables 14 and 15 have high values of R.

III. Amethopterin

A. Introduction

The action of amethopterin in inhibiting leukemia cell growth originates in its interference with DNA synthesis. Presumably, the amethopterin reacts with the enzyme dihydrofolic reductase and forms an irreversible or near irreversable complex with it. Tetrahydrofolic acid then becomes unavailable to the cells, which inhibits the biosynthesis of thymidylic acid and other precursors to nucleotides which appear in RNA and DNA. The synthesis of DNA is essential for all life, and particularly, for cell replication. Where cell division is more rapid, the absence of nucleic acids is more important. For this reason amethopterin is, selectively, more effective against leukemic cells.

B. Data on Multiple Dose Treatments Using Amethopterin

The mice were injected with leukemia and drug

FIGURE 6

A PLOT OF $LOG_{10}{}^{R}$ vs. TOTAL DOSE. NSC 409962-DATA SET II*
USING PARAMETERS OF MINIMUM POINT LISTED IN TABLE 11-
HIGH TOTAL DOSE POINTS OF DATA SET II ADDED

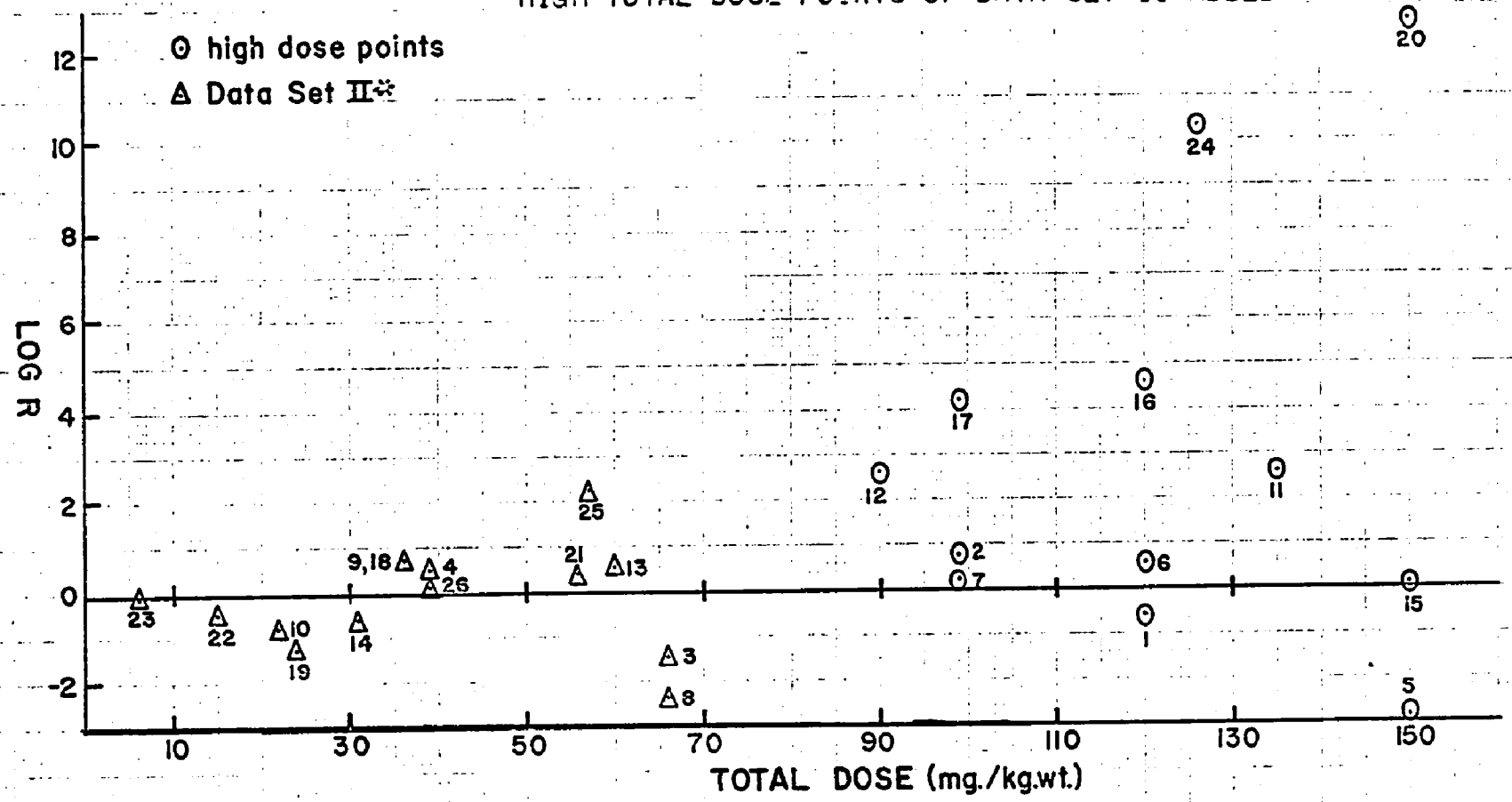


FIGURE 7

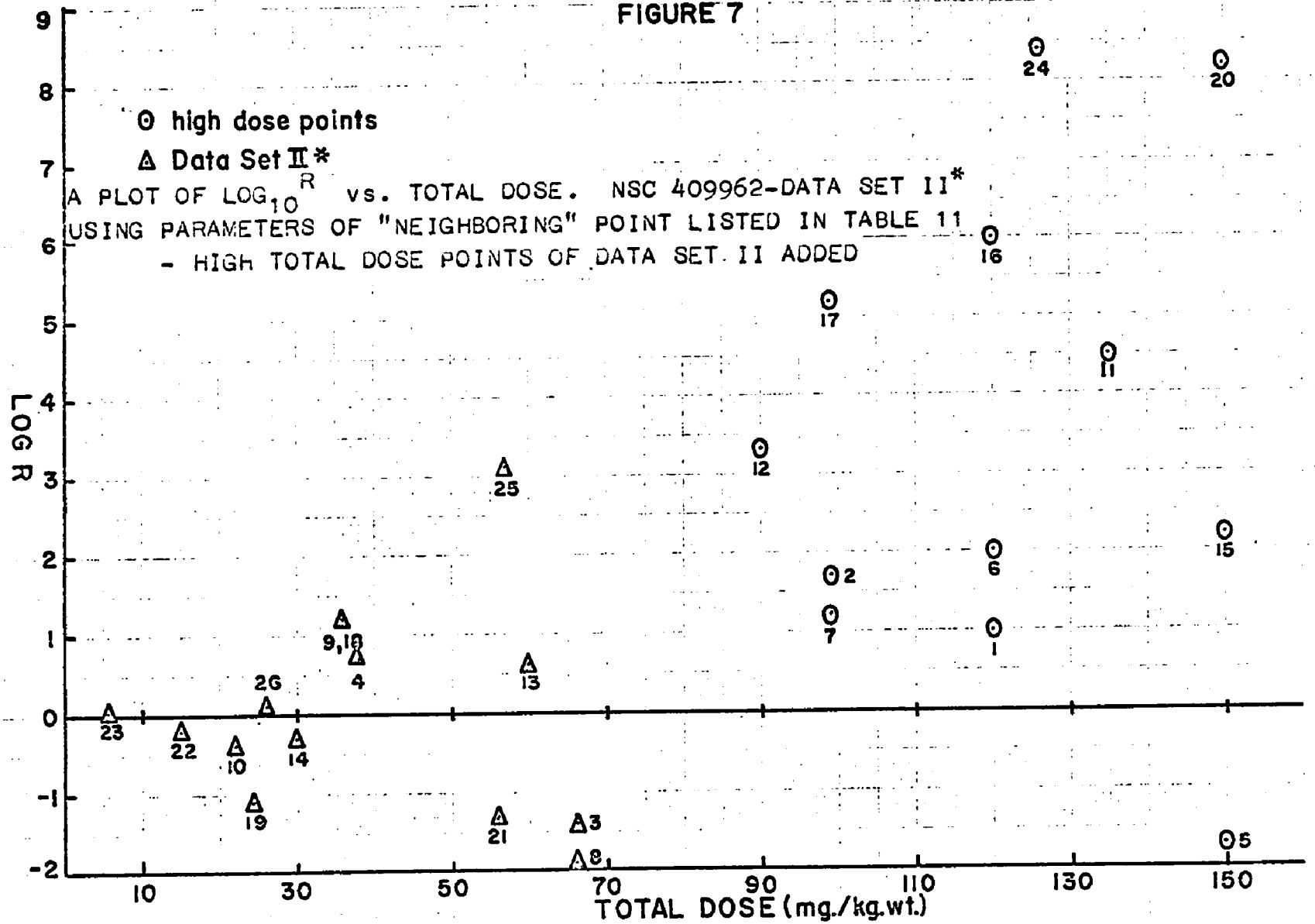

⊙ high dose points
Δ Data Set II*
A PLOT OF LOG10 R vs. TOTAL DOSE. NSC 409962-DATA SET II*
USING PARAMETERS OF "NEIGHBORING" POINT LISTED IN TABLE 11
- HIGH TOTAL DOSE POINTS OF DATA SET II ADDED
LOG R
TOTAL DOSE (mg./kg.wt.)
9
8
7
6
5
4
3
2
1
0
-1
-2
10
30
50
70
90
110
130
150
24
20
16
17
11
12
25
15
6
2
7
1
9,18
4
26
13
23
22
10
14
19
21
3
8
5

TABLE 14

VALUES OF N_k, R_k, LOG R_k, CALCULATED MINUS OBSERVED DAY OF DEATH FOR NSC 409962, DATA SET II, CELL MODEL I, USING PARAMETERS FITTED TO LOW DOSE POINTS LISTED IN TABLE 11 AS "MINIMUM POINT"

Experiment Number (k)	N_k	R_k	Log R_k	Calculated-Observed Day of Death
1	41.1	.523	- .281	- .42
2	1,502.2	3.090	.490	.73
3	1,029,397.6	.097	-1.013	-1.52*
4	97,828,030.8	2.202	.343	.51*
5	0.4	.011	-1.951	-2.93
6	41.1	2.429	.385	.58
7	1,502.2	1.434	.157	.23
8	1,029,397.6	.045	-1.346	-2.02*
9	74,041,616.5	2.910	.464	.70*
10	693,511,555.2	.311	- .508	- .76*
11	3.8	55.927	1.748	2.62
12	7,906.3	58.706	1.769	2.65
13	3,927,797.1	2.546	.406	.61*
14	230,558,980.1	.434	- .363	- .54*
15	0.4	1.119	.049	.07
16	41.1	1,127.337	3.052	4.58
17	1,502.2	665.669	2.823	4.23
18	74,041,616.5	2.910	.464	.70*
19	1,397,488,828.6	.154	- .812	-1.22*
20	0.2	385,221,904.500	8.586	12.88
21	108,237,092.0	1.990	.299	.45*
22	931,910,716.2	.498	- .303	- .45*
23	497,755,474.4	.933	- .030	- .05*
24	27.3	7,883,664.176	6.897	10.35
25	3,379,338.3	63.753	1.804	2.71*
26	296,887,790.1	1.563	.194	.29*

* These 14 points are included in Data Set II*.

TABLE 15

VALUES OF N_k, R_k, LOG R_k, CALCULATED MINUS OBSERVED DAY OF DEATH FOR NSC 409962, DATA SET II, CELL MODEL I, USING PARAMETERS FITTED TO LOW DOSE POINTS LISTED IN TABLE 11 AS "NEIGHBORING" POINT

Experiment Number (k)	N_k	R_k	Log R_k	Calculated-Observed Day of Death
1	4.2	5.096	.707	1.06
2	343.3	13.519	1.131	1.70
3	884,891.7	.113	- .947	-1.42*
4	67,756,703.5	3.179	.503	.75*
5	0.0	.324	- .489	- .73
6	4.2	23.657	1.374	2.06
7	343.3	6.275	.798	1.20
8	884,891.7	.052	-1.280	-1.92*
9	32,446,745.7	6.639	.822	1.23*
10	436,435,940.7	.493	- .307	- .46*
11	0.2	946.738	2.976	4.46
12	2,570.1	180.595	2.257	3.39
13	4,330,940.8	2.308	.363	.55*
14	133,985,784.4	.746	- .127	- .19*
15	0.0	32.422	1.511	2.27
16	4.2	10,981.206	4.041	6.06
17	343.3	2,912.799	3.464	5.20
18	32,446,745.7	6.639	.822	1.23*
19	1,234,722,718.4	.174	- .758	-1.14*
20	348.5	286,919.194	5.458	8.19
21	1,433,201,198.5	.150	- .823	-1.23*
22	620,689,277.2	.747	- .126	- .19*
23	432,808,168.9	1.072	.030	.05*
24	538.9	399,740.145	5.602	8.40
25	1,888,357.9	114.090	2.057	3.09*
26	396,783,793.0	1.169	.068	.10*

* These 14 points are included in Data Set II*.

was injected daily as described in Section I of this chapter. Eighteen experiments were carried out and the dose level, number of treatments, and average day of death are presented in Table 16 (p. 69). Experiments 1-13 involved ten mice in each set, 14-18 involved only six to a set. In the first thirteen experiments animals were treated for 15 days if they lived that long, while in the last five they were treated until death.

The parameters for minimum Q_5 using Cell Model I are given in Table 17. Cell Model IR yielded much higher values of Q_5 and is obviously not applicable to these experiments. These parameters are used to calculate values of R_k (Table 18) and plotted against total dose in Figure 8.

TABLE 17

PARAMETERS OBTAINED FOR AMETHOPTERIN DATA LISTED IN TABLE 16 BY LEAST SQUARES MINIMIZATION, CELL MODEL I

Value	Cell Model I
μ	.701386
α^2	1.60317
β	-.07331
Q_5	3.21381

The values of μ and α^2 were found for fixed values of β by minimizing Q_5. Graphical representation of the result is given in Figure 9. Also, a plot of Q_5 versus β at the minimum is presented in Figure 10.

TABLE 16

AMETHOPTERIN DATA

Experiment Number	Dose in mg/kg.wt.	Number of Treatments	Average Day of Death
1	0.3	8	9
2	0.5	10	11.5
3	0.5	12	13
4	0.67	13	14.5
5	0.75	15	17
6	0.75	11	12
7	1.0	15	17.5
8	1.0	15	16.5
9	1.12	14	15
10	1.5	15	18.5
11	1.7	15	17.5
12	2.0	15	19
13	2.25	15	19.5
14	1.0	20	21
15	1.0	17	18.5
16	1.0	20	21
17	1.0	23	24
18	1.0	22	23

TABLE 18

VALUES OF N_k, R_k, LOG R_k, CALCULATED MINUS OBSERVED DAY OF DEATH FOR AMETHOPTERIN - CELL MODEL I, USING PARAMETERS LISTED IN TABLE 17

Experiment Number (k)	N_k	R_k	Log R_k	Calculated-Observed Day of Death
1	39,959,775.1	5.391	.732	1.10
2	81,515,260.3	1.226	.089	.13
3	318,085,883.6	.677	- .169	- .25
4	217,285,316.9	.460	- .337	- .51
5	330,703,009.6	.140	- .853	-1.28
6	53,083,294.4	4.058	.608	.91
7	88,121,433.3	.244	- .612	- .92
8	88,121,433.3	1.134	.055	.08
9	40,423,281.1	5.329	.727	1.09
10	11,585,546.7	.400	- .397	- .60
11	5,951,647.5	3.619	.559	.84
12	2,435,197.5	.884	- .053	- .08
13	1,248,556.4	.800	- .096	- .14
14	183,074,360.6	1.176	.071	.11
15	134,618,583.6	.742	- .129	- .19
16	183,074,360.6	1.176	.071	.11
17	168,723,807.2	1.276	.106	.16
18	181,014,829.9	1.190	.076	.11

A PLOT OF LOG R vs. TOTAL DOSE FOR AMETHOPTERIN DATA USING CELL MODEL I AND PARAMETERS OF TABLE 17

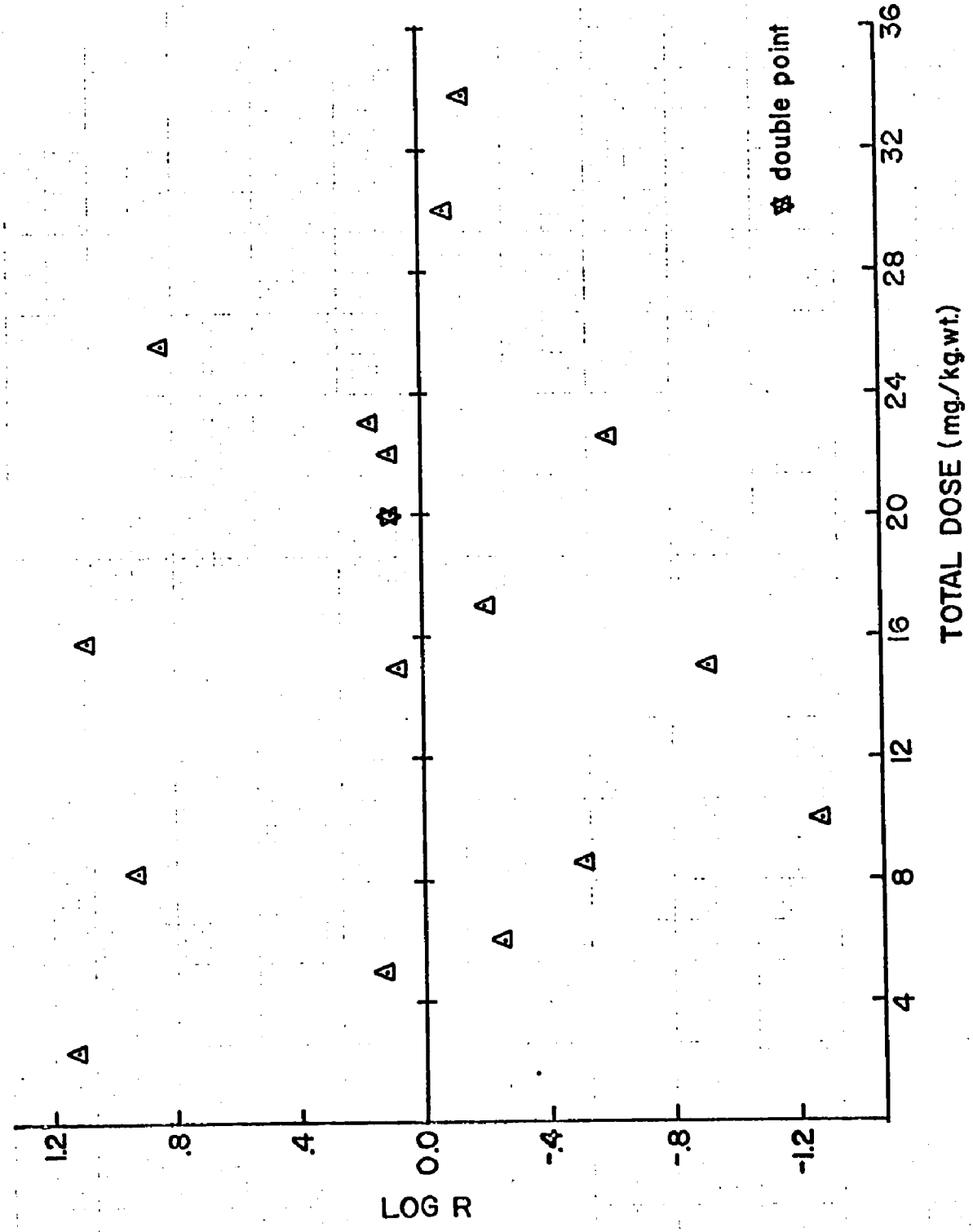


FIGURE 8

FIGURE 9

A PLOT OF μ vs. β FOR AMETHOPTERIN DATA OF TABLE 16 USING CELL MODEL I AND THE LEAST SQUARE CRITERION Q_5. THESE ARE BEST VALUES OF μ (and σ^2) AS FUNCTIONS OF β WITH α FIXED AT 1.53506

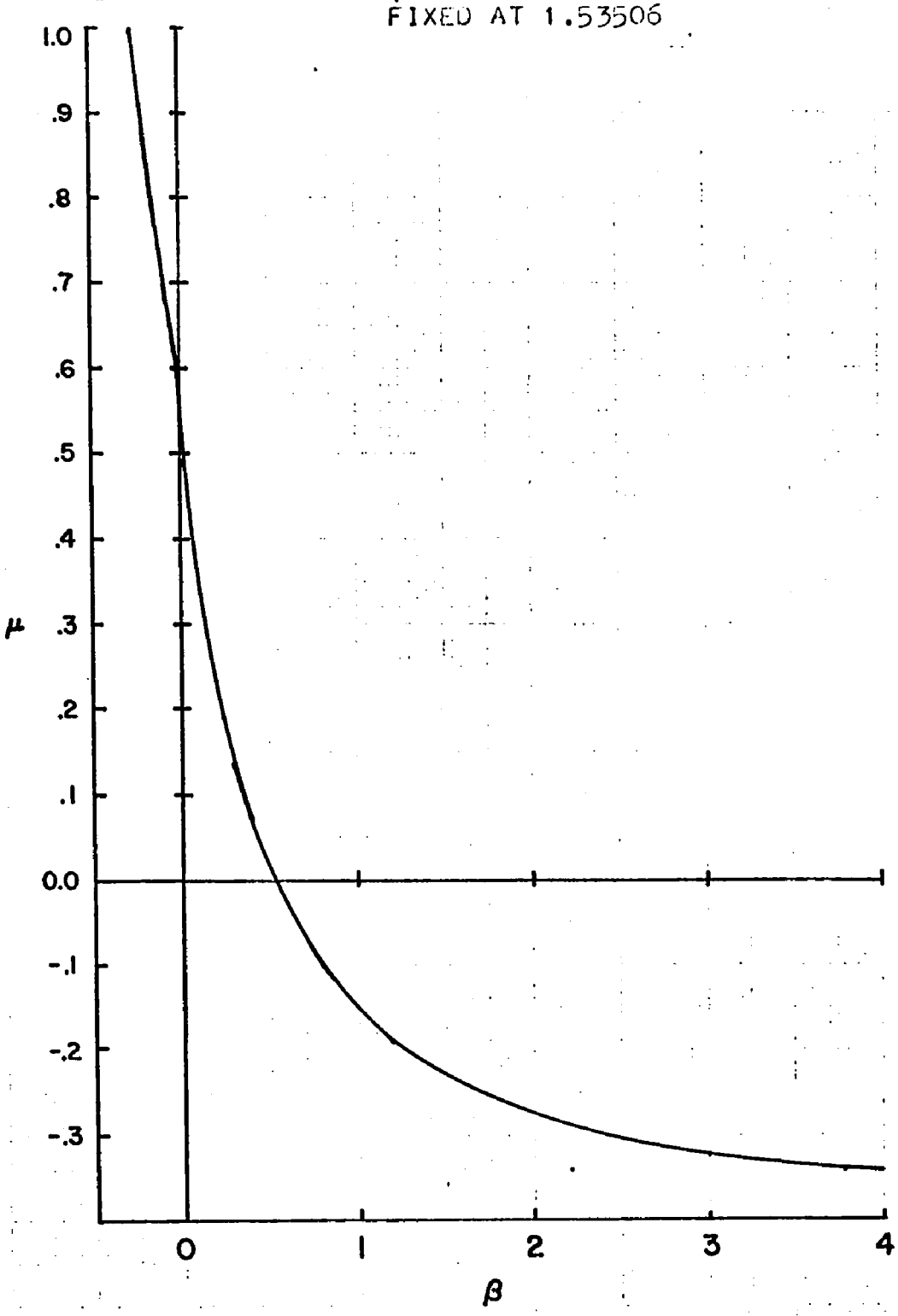
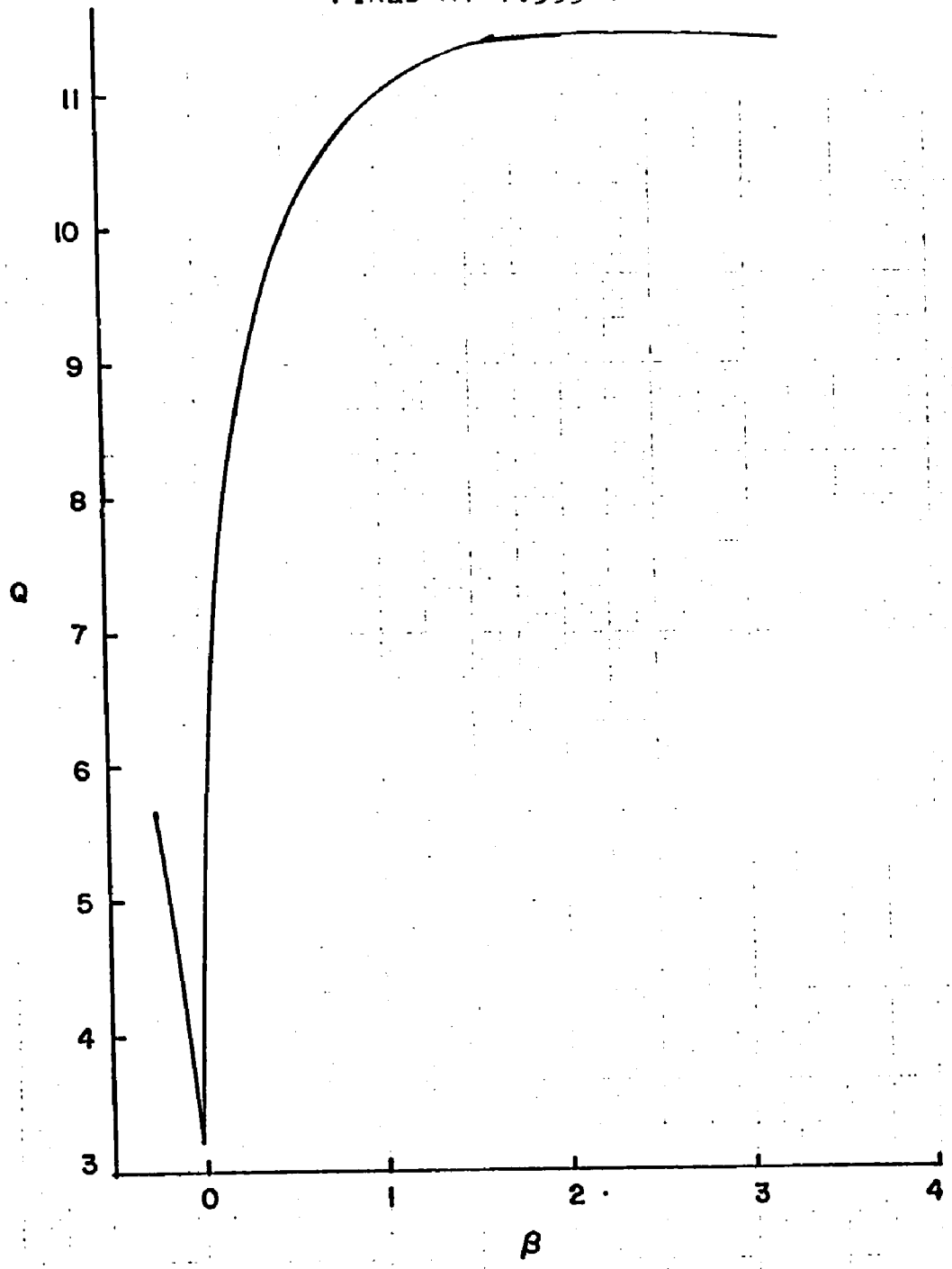


FIGURE 10

A PLOT OF Q_5 vs. β FOR AMETHOPTERIN DATA OF TABLE 16 USING CELL MODEL 1. THE Q_5 VALUE IS THAT OBTAINED WITH THE BEST VALUES OF μ AND σ^2 AS A FUNCTION OF β, WITH α FIXED AT 1.53506



Cell Model I does not permit negative β. Yet, $\beta = -.07331$ is a sharp minimum point. This is a case where the information gained may lead to further insight into the problem. A negative β is a result outside of the spirit in which the model was concocted, but, nevertheless, this result has an interpretation which will be discussed in Chapter V. The model anticipated only a partial accumulation of the drug which corresponds to a positive β. A negative β implies that more drug appears in the host than was actually injected.

CHAPTER IV

DISCUSSION PART I - GENERAL

While a set of "best" parameters have been found for a model of multiple drug treatments of leukemic mice using, a) NSC 409962, and b) amethopterin, the reliability of the parameters, or lack of reliability, is not immediately obvious. The estimation of errors in parameters are easily considered in linear regression models (10); for nonlinear equations such as the models considered here, no general analysis of errors is available. The extent to which a good or bad fit is a consequence of an accidental arrangement of experiments rather than the correctness of the analysis must be considered. We will treat a number of questions of this character in what is to follow. The purpose of this is twofold, first to assess the confidence to be given to the results, and secondly, to indicate, at least qualitatively, some defects of past studies and to suggest improvements to be incorporated in future experimental design.

The data of Skipper et al., (34) which have been analyzed in the preceeding chapters contain large fluctuations. If the least squares function Q_5 to be minimized is insensitive to a change in one of the parameters, it is evident that the normal fluctuations in experimental data could change the

minimum in Q_5 by a small amount while changing this parameter by a relatively large amount. In those cases where Q_5 exhibits this character, experimental fluctuations may easily change the outcome. On the other hand, if Q_5 has a sharp minimum it is less likely that random errors in the data would have a large effect on the values of the parameters obtained for the minimum. Examples of both sharp and flat minima have been obtained here; in Figure 10 (Table 17), Q_5 varies rapidly with β at the minimum and in Tables 6 and 7, Q_5 is insensitive to β. The problem is even more general. Even when Q_5 varies rapidly with each of the parameters at the minimum (the others being fixed), the minimum may be quite flat in a direction where two or more parameters change together. In such a situation, the numerical values of the parameters may be unreliable, and near-minimum values of the least squares function are found with many sets of parameters. This difficulty is present in our current study, but has not been analyzed. An extensive general discussion of nonlinear minimization can be found in reference (11).

The hypothesis of a resistant strain of leukemic cells has been suggested by Skipper, et al., (32), and demonstrated in a series of experiments by Law (24). We have incorporated this idea in our calculations, and it has been useful on the NSC 409962 experiments using Data Set I but not those using Data Set II. In the spirit of the present analysis a resistant strain is taken to have a log normal tolerance distribution with a mean and variance, and a certain probability

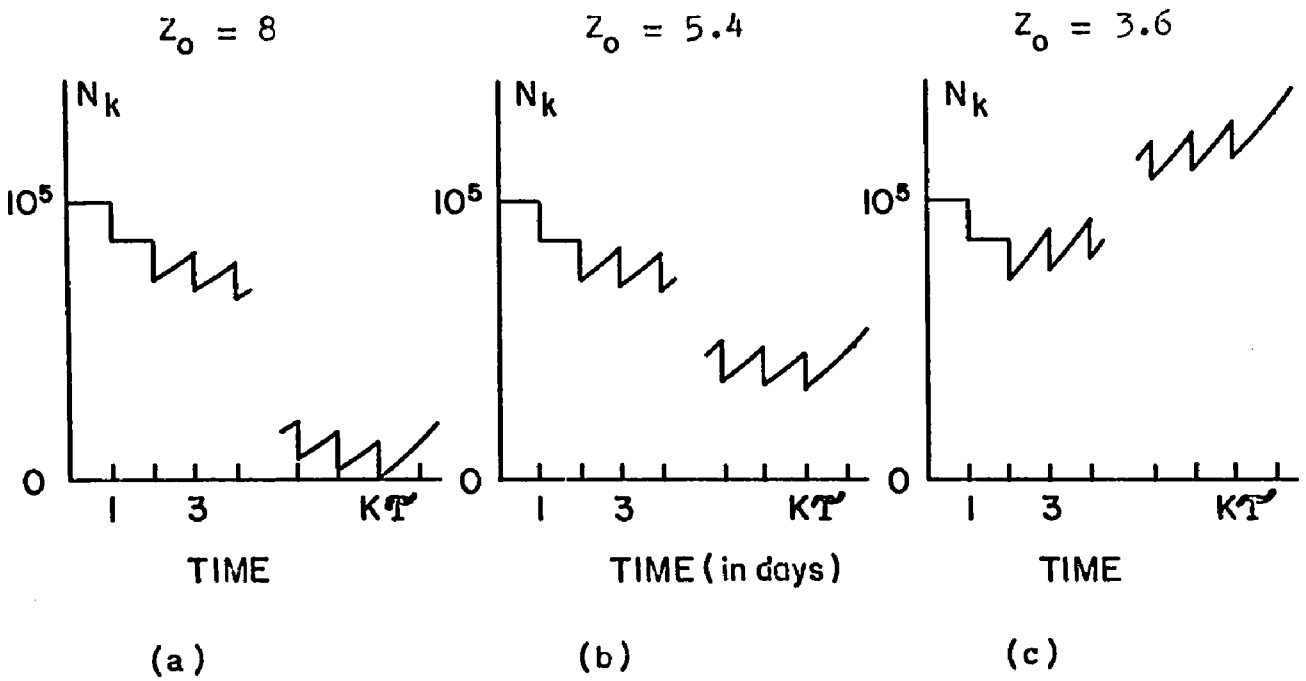
of formation as a mutant. The mean μ_r is large, the variance σ_r^2 is small. Both are fixed quantities so chosen that cell kill of the resistant strain occurs rarely if at all. P, the mutation probability, is variable. This must necessarily, lead to a Q_5 which is equal to or less than in the model without a resistant strain, an equality arising when P = 0. The experiment is insensitive to μ_r and σ_r^2 of the resistant strain provided that μ_r is sufficiently large.

Not all data are suitable for <u>detection</u> of the resistant strain. Since the mutation rate P is small, of the order of 10^{-5} or 10^{-6}, the resistant strain is a trivial fraction of the leukemic cell population unless most of the non-resistant cells are wiped out. Accordingly, the presence of a resistant strain is evident only in those experiments in which large doses of the drug are used. In Figure 11, a-c, where the number of leukemic cells is sketched versus time for the three different doses used in Data Set I, the resistant strain is important in 17a only. This is also seen by looking at the N_k values in Table 3 and by comparing Figures 3 and 4. In Data Set I, Q_5 was reduced from 42.05 to 25.1 (Table 2) by introduction of the assumption of a developing resistant strain of cells. The variance of the tolerance distribution decreases as might be expected, because the omission of the resistant strain in the model, but not in fact, artificially broadens the tolerance distribution.

The resistant strain becomes important at high dose. However, further doses are ineffective in elimination of

FIGURE 11

SKETCHES OF POPULATION SIZE VERSUS TIME



resistant cells, and, therefore, the drug treatment appears to be decreasingly effective as more drug is given. This is most easily seen if the same dose is repeated a different number of times in parallel experiments. Data Set 1 is well designed for this, and, indeed, it is with this set that the significance of a resistant strain becomes apparent.

In all three data sets, treatments were given daily. For determination of drug storage, an experimental design with variable spacings of drug application would be useful. Such experiments are not available in a useful form in chemotherapy of L1210 leukemic mice using the drugs analyzed here. For example with NSC 409962 drug seems to disappear largely within a day. Accordingly, the drug storage cannot be estimated with

any degree of confidence if the doses are applied daily. For example if β were approximately equal to 4, a comparison of experiments where the treatments are given 2, 4 or 8 times a day would be very useful in its determination. Any new experimental design should incorporate such a procedure.

Toxicity, that is, killing of the experimental animal by drug treatment, obviously interferes with the analysis of the problem since this may be mistaken for death from leukemia. Toxic effects of these drugs are well known, and occur at high total dose. They are seen in the statistical analysis as an apparent decreased drug efficiency at high dose, that is, large values of R. Both a resistant strain of leukemic cells, and toxicity in the experimental animal act in the same direction, but do not depend on the parameters in the same way. It was shown in Tables 6 and 7 that the introduction of a resistant strain did not change the results for Data Set II. However, if the high total-dose points of this set were eliminated, subset II* yielded a lower Q and different parameters μ, α^2, and β. The high dose points when fitted to these parameters yielded mostly large R (Figures 6 and 7) which is consistent with, but not proof of, toxicity. Toxicity was demonstrated in laboratory experiments at high total dose (34).

The results of applying Cell Model I (no resistance) to amethopterin data are satisfying in that the lowest value of Q_5 found in this study and the sharpest minimum with respect to drug storage was obtained. However, the results, as

shown in Table 17, are incorrect because the negative value of β means that the drug is being manufactured in the animal. The possible causes of this unacceptable result are discussed separately in Chapter V. This appears to be the most interesting information found from the laboratory experiments treated herein, and it is hoped that the subsequent discussion will illuminate the problem.

CHAPTER V

DISCUSSION PART II - AMETHOPTERIN

I. Introduction

The best fit among all sets of data analyzed here and using the Q_5 least squares criterion was obtained on chemotherapy experiments using amethopterin. The Q_5 value using Cell Model I is low ($Q_5 = 3.21$), and at the point where the best parameters (μ, σ^2, and β) are obtained, the variation of Q with β (Chapter III, Figure 10) and with μ is sharp. However, at this point β equals -.07331, a result which appears to state that the amount of drug in the animal increases after injection.

This result is absurd, and yet the fit of the model to the data is excellent. Evidently, certain qualities of the real system are not incorporated in the model. Hence we looked for properties of the interaction between drug and leukemic cells which have been omitted from the model and yet would look like a negative β in Cell Model I in multiple dose experiments. Some of these are:

1. Cells which have been exposed to drug are weakened, and are killed more easily by later drug application.

2. The growth rate of cells is not a constant. This may decrease at high cell concentration, or may be perturbed by chemical action of earlier drug treatment.

3. The action of the drug is delayed; any given application of the drug becomes effective only after some time has passed.

The reason why each of the three enumerated modifications listed above might appear as a negative β is that the drug shows an apparent increase in effectiveness with time in each case. If it is assumed, as in Cell Model I, that there is no change in drug effectiveness with time, this effect may appear in the model as an apparent addition of drug which has not, in fact, been applied. This apparent addition of drug would appear as a negative β.

II. A Simple Continuous Model

In order to illuminate this problem, a simple continuous model is here formulated. This is not intended to replace the more accurate statistical model, but merely to give insight into the problem. It will also demonstrate that a β value near zero is imperative for this collection of experiments.

In this simple continuous model, the dose D is added at a steady rate a. The fractional rate of inactivation of drug per unit dose is given by β. Accordingly, the buildup of drug in the animal is given by

$$\frac{dD}{dt} = a - \beta D \qquad (1)$$

Since at $t = o$, no dose is present, one finds on integration

$$D = \frac{a}{\beta}(1-e^{-\beta t}) \qquad (2)$$

Coupled to this result is the equation for cell multiplication. The rate of formation of cells is proportional to the number present. The constant of proportionality is the natural growth rate α minus some monotonically increasing function of the dose. This may be written

$$\frac{dn}{dt} = n(\alpha - f(D)) \qquad (3)$$

where $f(D)$ is undefined. At this point, the simple substitution $f(D)$ equals kD is made. This is largely based on a desire for simplicity, but, in fact, it differs only slightly from the low dose side of the tolerance distribution of the statistical models proposed earlier. Upon integration, Equation (3) leads to

$$n = n_o \exp\left[(\alpha - \frac{ka}{\beta})\, t - \frac{ka}{\beta^2}(e^{-t\beta} - 1)\right] \qquad (4)$$

For positive β, and large t, this reduces to

$$n = n_o \exp\frac{ka}{\beta^2} \exp\left[(\alpha - \frac{ka}{\beta})t\right] \qquad (5)$$

We distinquish three cases:

a) $\alpha - \frac{ka}{\beta} < 0$

b) $\alpha - \frac{ka}{\beta} = 0$

$$c) \quad \alpha - \frac{ka}{\beta} > 0 \qquad (6)$$

Only case (a) leads to a "cure". The course of cell growth begins at some small number, increases to a maximum and then goes to zero. If this maximum is less than the lethal number of cells, a cure is obtained, if it is greater, the animal dies before the drug is able to reduce the cell count. Case (b) leads to an equilibrium situation, i.e., assuming that

$$n_e = n_o \exp\left(ka/\beta^2\right) \qquad (7)$$

is less than the lethal cell count. Case (c) represents exponential growth of cells and certain death of host.

In studying the experimental data on amethopterin (Table 16), it is seen that the drug dose of 1 mg/kg.wt. is in a special category. At this dose level, applied daily, the mice live indefinitely (for a long time) until the treatment is stopped. When this happens, the mouse dies one day later. If the dose is larger, the mice live for several days after the drug treatment is stopped. If the dose is smaller, the mice do not survive but die during the treatment. This indicates that the number of cells in the animal remain constant for a long time (apparent equilibrium), that is $\alpha \approx ka/\beta$. On the other hand, this constant number is very near the lethal number, n_e . Accordingly,

$$\frac{n_e}{n_o} = 10^4 \approx \exp\left(\frac{ka}{2}\right) \qquad (8)$$

On substitution of $\alpha = \frac{ka}{\beta}$, one finds on using the experimental value of $\alpha = 1.53$, that $\beta = 1/6$.

If it is assumed that $\beta < 1/6$ (this includes $\beta = 0$ or negative), the cell count goes through a maximum and then decreases. The amethopterin experiments using a dose of 1 mg/kg.wt. shows that the animal is within a day of death for many days. This means that β is near 1/6. At $\beta = 0$, it is easy to show that the maximum in number of leukemic cells as a function of time is very flat as the data demands. Equation (4) at $\beta = 0$ reduced to

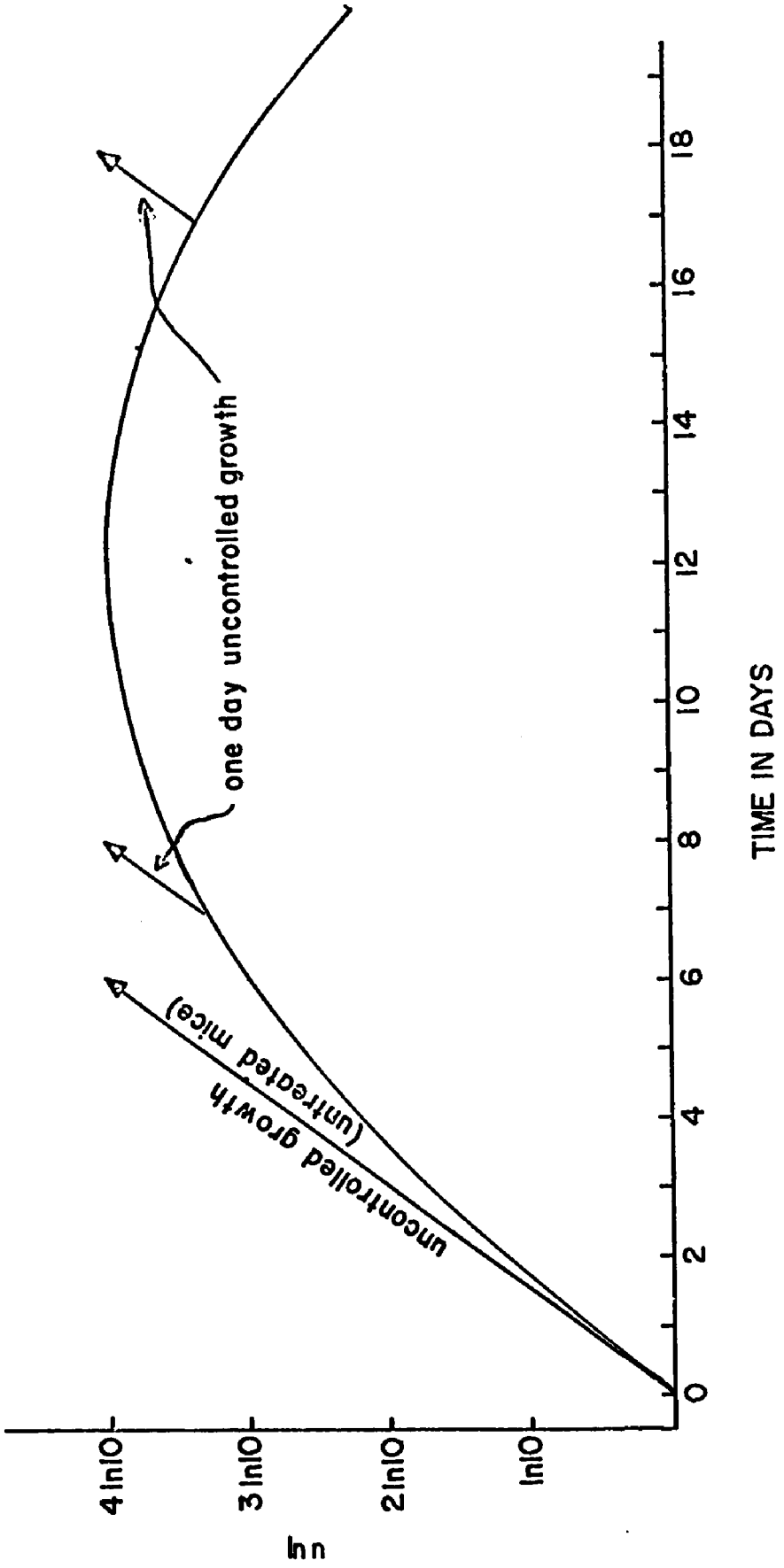
$$\ln \frac{n}{n_0} = \alpha t - kat^2/2 \qquad (9)$$

If n_{max} is taken equal to 10^9, $t_{max} = \frac{\alpha}{ka} = 12$. A plot of $\ln(\frac{n}{n_0})$ is shown in Figure 12. It is easily seen from this graph that the number of cells is within one day's uncontrolled growth of 10^9 when t lies between 7 and 17 days.

These considerations indicate that β values near zero are entirely consistent with the amethopterin data using this simple continuous model. Values of β much greater than 1/6 ($\beta = 1$ or 2) could not fit these experiments. In this context the value of $\beta = -.07331$ found using Cell Model I is seen to be sensible. The cause of the negative β, (which means that drug appears in the host faster than it was actually injected), however, lies in a defect of the model. Some possible deficiencies have been mentioned earlier in this chapter and further discussion of the negative β problem follows.

FIGURE 12

IN GROWTH vs. TIME FOR $\beta = 0$. CONTINUOUS MODEL.
AMETHOPTERIN DATA



III. Search for An Explanation of Negative β in Amethopterin Experiments

A. One possible source of negative β using Cell Model I is that the growth rate of the cells is not constant as assumed, but diminishes with increased drug concentration. An example where growth rate is not necessarily a constant was cited by Cronkite (8). In this study Ehrlich ascites tumor cells grow exponentially for four days and thereafter the growth rate diminishes. At later time intervals, the growth may be negative, that is cells die faster than they are being produced.

Cell Model I was amended to allow the growth rate to diminish with drug concentration from its value in untreated populations. The formulation chosen was

$$\alpha = \alpha_0 \exp(-\uparrow Z_k).$$

α_0 was taken to be 1.53506, the value used for α in the previous calculations. By introducing an extra parameter it is always possible to improve the fit of the model. In Table 18, the outcome of minimization of Q_5 is presented for this model. β is still negative.

In this minimization the value of $\uparrow$ is close to zero, which means that α varies little from α_0 and the other parameters are essentially unchanged (see Table 17).

B. Another possible source of an apparent negative β is that the mean of the tolerance distribution might decrease with increased dosing, reflecting a weakened, more susceptible

TABLE 19

PARAMETERS OBTAINED FOR AMETHOPTERIN DATA LISTED IN TABLE 16 BY LEAST SQUARE MINIMIZATION USING $\alpha = \alpha_0 \exp(-\gamma Z_k)$, $\alpha_0 = 1.53506$

Parameter	Value
μ	.716786
σ^2	1.669570
β	-.075970
γ	.000413
Q_5	3.20226

population. Accordingly, the assumption is made that the mean of the tolerance distribution decreases exponentially with dose level, i.e.,

$$\mu = \mu_0 \exp(-\delta Z_k).$$

The minimization of Q_5 using this modification of Cell Model I by the Direct Search method led to the parameters listed in Table 18.

TABLE 20

PARAMETERS OBTAINED FOR AMETHOPTERIN DATA LISTED IN TABLE 16 BY LEAST SQUARE MINIMIZATION USING $\mu = \mu_0 \exp(-\delta Z_k)$

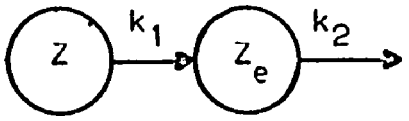
Parameter	Value
α	1.53506
μ_0	8.60137
σ^2	4.90317
β	-.04125
δ	.262
Q_5	2.46029

These values had not converged to a minimum when the computation was terminated; the trend of the apparent best parameters indicates that the results are far from realistic. The large value of δ indicates a great change in the mean tolerance of the leukemic cell population as a function of the drug concentration. The value of μ_o in Table 20 (the $\log_{10}$ of the mean tolerance of the population) is eight orders of magnitude greater than the log mean tolerance found using Cell Model I, where μ is assumed independent of drug concentration. On the other hand if the cell dose is equal to 1 mg/kg.wt., the mean, μ, becomes equal to 0.7 (approximately the μ value of Cell Model I), after eight doses. This decrease in mean tolerance of the leukemic cell population of 10^8 as a function of drug concentration is manifestly absurd. β is still negative.

C. It is conceivable that the "effective" drug concentration is delayed for some time after application. This may be caused by biochemical reactions in the host (or cell) which alter and delay the effect of the drug. Plackett and Hewlett in the two-drug problem treat and account for the disparity between applied and "effective" drug. Their formulation is not time dependent (29). For amethopterin there is biochemical evidence that there is indeed a time delay, because amethopterin reacts with the available folic acid reductase. Further amounts of folic acid reductase are slowly generated and combine with the next dose of the drug.

A Simple Chemical Mechanism of Delayed Action

Let Z be the applied drug and Z_e stand for effective drug. Let k_1 be the rate of conversion of Z to Z_e, while k_2 represents the rate of decay of Z_e, as here shown:



The differential equations are:

$$\frac{dZ}{dt} = -k_1 Z \qquad (1)$$

$$\frac{dZ_e}{dt} = k_1 Z - k_2 Z_e \qquad (2)$$

Equation (1) has the solution

$$Z = Ce^{-kt} \qquad (3)$$

The boundary conditions for the problem are that an application of drug is given at $t = 1, 2, 3...$etc. Accordingly, the particular solution desired is a sum of solutions of the type of Equation (3) and becomes

$$Z(t) = \sum_{n=0}^{t-1} Z_o e^{-k_1 n} \quad \text{with } t \text{ an integer.} \qquad (4a)$$

For t non-integral, one obtains

$$Z(t) = Z([t]) e^{-k_1} \qquad (4b)$$

where $[t]$ signifies the maximum integer not greater than t. The solution for Equation (2) taking $Z_e(0) = 0$, and $Z(0) = Z_o$ is

$$Z_e(t) = \frac{k_1 Z_o}{k_2 - k_1} (e^{-k_1 t} - e^{-k_2 t}) \qquad (5)$$

The solution for $Z_e(t)$ arising from an application of drug Z_o at $t = \gamma$ is

$$Z_e(t;\gamma) = \frac{k_1 Z_o}{k_2 - k_1} (e^{-k_1(t-\gamma)} - e^{-k_2(t-\gamma)}) \qquad (6)$$

The total quantity $Z_e(t)$ is obtained by sum of daily doses

$$Z_e(t) = \sum_{\gamma=1}^{[t]} Z_e(t;\gamma)$$

This variation of Cell Model I involves the use of two new parameters k_1 and k_2 instead of the original β, a net increase of one parameter. The results of minimization are given in Table 21.

The Q_5 value is small. The values of k_1 and k_2 are positive, which is equivalent in a sense to a positive β in Cell Model I. The value of μ, the mean of the <u>effective</u> dose, has decreased sharply by comparison with the tolerance distribution, has decreased sharply by comparison with the values shown for Cell Model I in Table 17. This is a measure of the fact that the effective doses computed with this model

TABLE 21

PARAMETERS OBTAINED FOR AMETHOPTERIN DATA LISTED IN TABLE 16 BY LEAST SQUARES MINIMIZATION USING "DELAYED DRUG ACTION"

Parameters	Values
μ	-.185845
σ^2	1.66867
k_1	.105757
k_2	.1161538
Q_5	2.8410

are very much smaller than the doses in Cell Model I with the best parameters. See the comparison in Table 22 of effective dose in this model and in Cell Model I. It is interesting to note that despite the substantial change in μ, the variance σ^2 remains essentially the same. The low value of k_2 indicates that the drug remains for a long time. However the delay in forming the effective drug leads to a drug increment pattern not very different from Cell Model I. This is illustrated in Figure 13 where the scales are chosen so that the mean tolerance to effective drug in each model has the same ordinate. Table 23 contains the computed values of R_k ($R_k = 10^9/N_k$ (calc.)) and Figure 14 shows a plot of log R_k versus total dose. Both of these use the parameters of Table 21 for the "delayed-drug action" modification of Cell Model I.

Let us look again at Table 22 where the effective dose after, say, 20 treatments at dose Z_o = 1 mg./kg.wt. is presented. In the original Cell Model I this represents an

TABLE 22

COMPARISON OF EFFECTIVE DOSE AT TIME OF EACH TREATMENT FOR "DELAYED-DRUG ACTION" VARIATION OF CELL MODEL I (USING PARAMETERS OF TABLE 21 AND FOR THE ORIGINAL CELL MODEL I (USING PARAMETERS OF TABLE 27)
Z_o = 1 mg/kg.wt.

Effective Dose Delayed-Action Model	Effective Dose Cell Model I
0.0	1.000
0.094	2.076
0.264	3.233
0.491	4.479
0.762	5.820
1.066	7.263
1.392	8.815
1.733	10.486
2.081	12.284
2.432	14.218
2.781	16.300
3.124	18.539
3.460	20.950
3.785	23.543
4.098	26.334
4.399	29.337
4.686	32.569
4.959	36.046
5.218	39.788
5.462	43.814
5.692	48.147
5.909	52.809

TABLE 23

VALUES OF N_k, R_k, LOG R_k, CALCULATED MINUS OBSERVED DAY OF DEATH FOR AMETHOPTERIN USING "TIME-DELAY" MODEL AND PARAMETERS LISTED IN TABLE 21

Experiment Number (k)	N_k	R_k	Log R_k	Calculated-Observed Day of Death
1	50,473,425.6	4.268	0.630	0.94
2	96,479,028.5	1.036	0.015	0.02
3	319,178,304.1	0.674	-0.170	-0.25
4	210,965,700.8	0.474	-0.324	-0.48
5	287,371,039.7	0.161	-0.791	-1.18
6	62,031,426.0	3.473	0.540	0.81
7	80,572,202.8	0.267	-0.572	-0.85
8	80,572,202.8	1.241	0.093	0.14
9	40,159,318.2	5.364	0.729	1.09
10	11,562,309.9	0.401	-0.396	-0.59
11	6,124,600.3	3.517	0.546	0.81
12	2,614,990.0	0.823	-0.084	-0.12
13	1,385,435.8	0.721	-0.141	-0.21
14	162,881,009.8	1.322	0.121	0.18
15	114,548,095.8	0.872	-0.058	-0.08
16	162,881,009.8	1.322	0.121	0.18
17	196,621,543.8	1.095	0.039	0.05
18	187,544,157.8	1.148	0.060	0.09

FIGURE 13

EFFECTIVE DOSE vs. TIME FOR AMETHOPTERIN DATA
(TABLE 21)

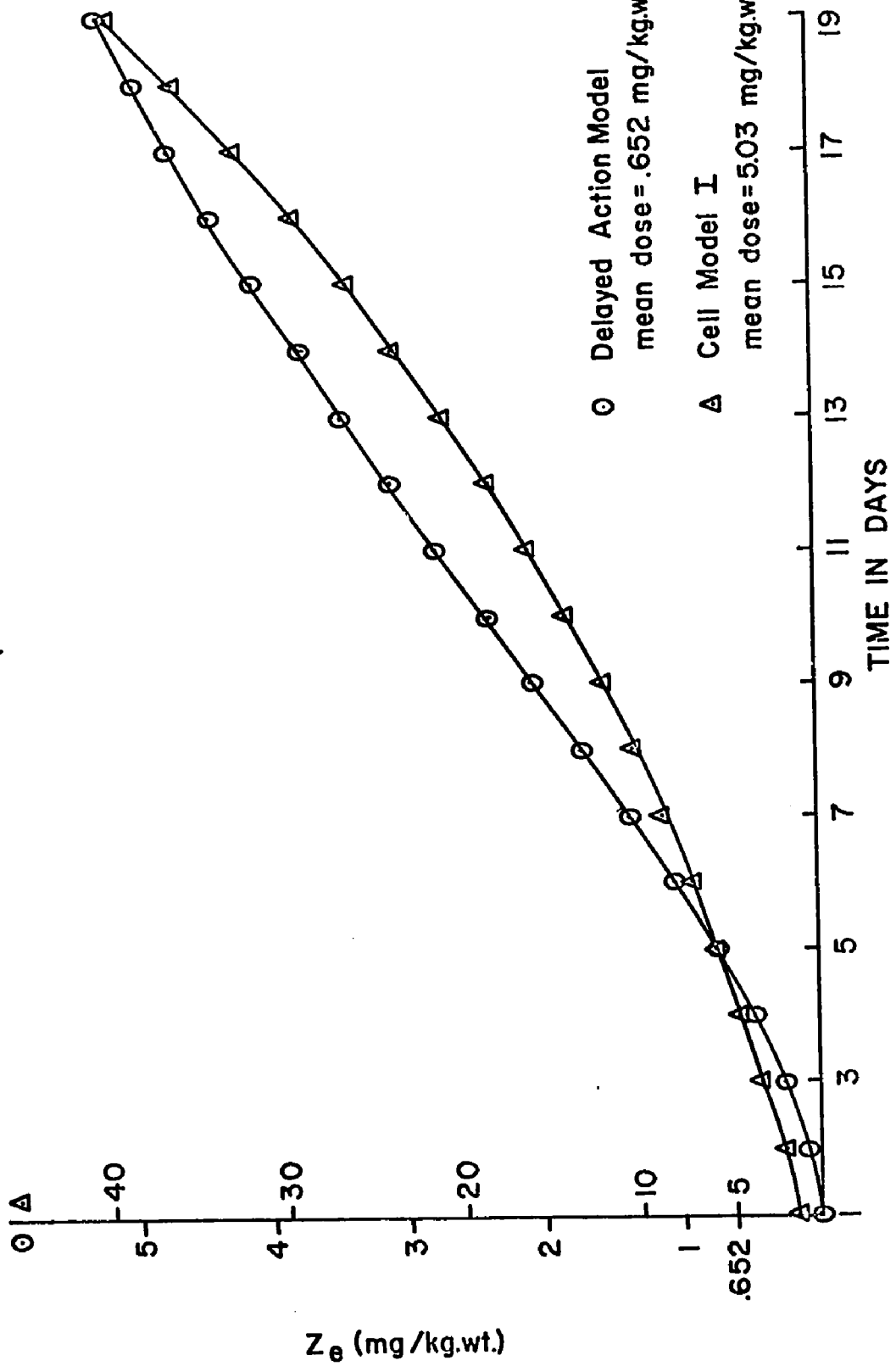
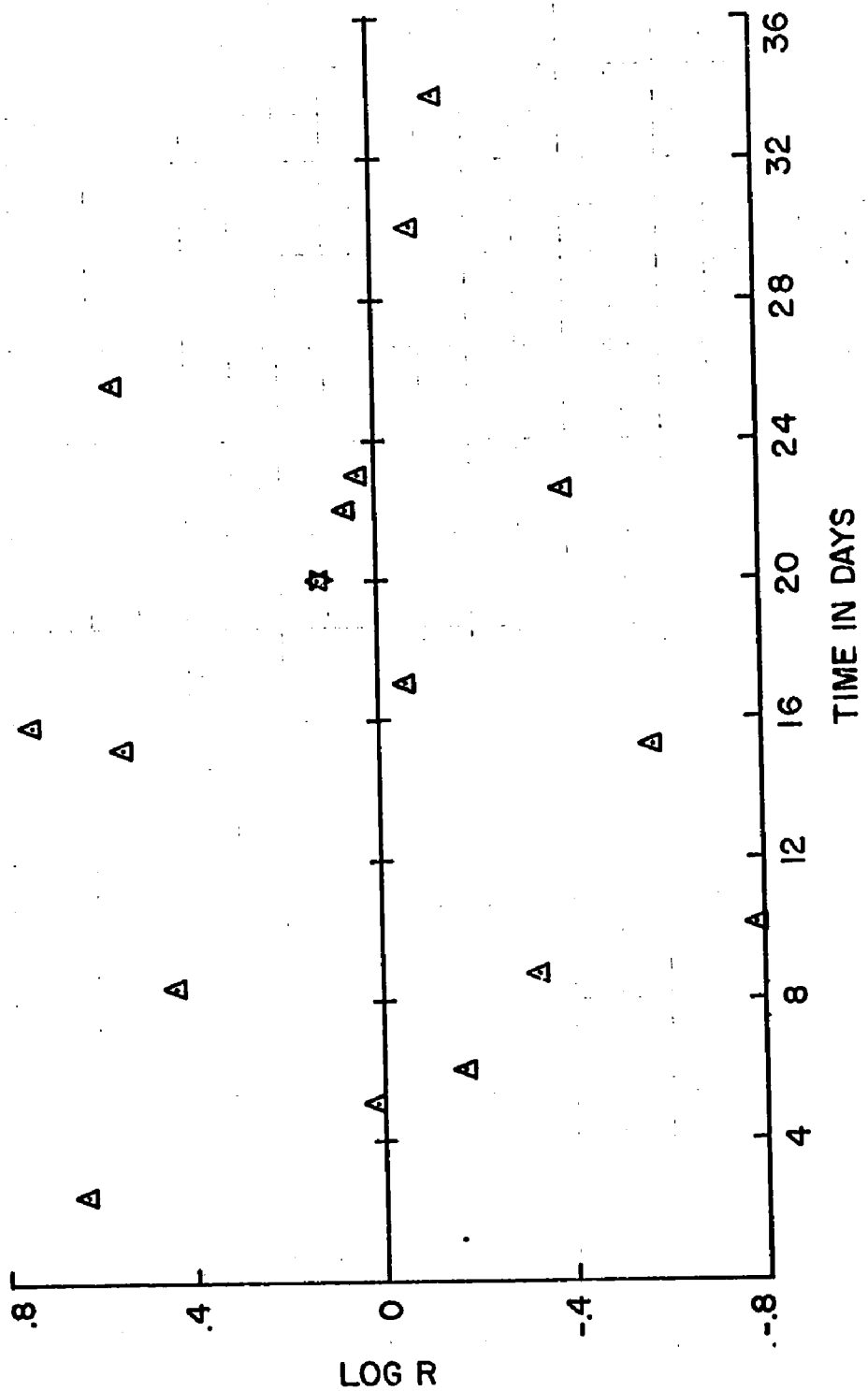


FIGURE 14

A PLOT OF $LOG_{10}R$ vs. TOTAL DOSE FOR AMETHOPTERIN DATA
(TABLE 23) FOR "DELAYED DRUG ACTION" MODEL
(PARAMETERS OF TABLE 21)



effective drug of 43.814 mg even though only 20 mg/kg.wt. had been given. This absurd result has been discussed before. In the delayed-drug action variation of the model the effective drug is 5.462 mg/kg.wt., representing about 27% of applied drug. This conforms reasonably well with the fact reported by Jacquez (21) that 60-80% of amethopterin is excreted in the urine of the mouse.

An interesting, possibly related fact is that toxicity (not drug action) of amethopterin given in daily doses has been reported to be more than cumulative in effect (34). If the effective drug were actually building up more rapidly than drug is applied (i.e. a negative β in Cell Model I, or a delayed-drug action) this pattern of toxicity might be anticipated.

CHAPTER VI

CONCLUSION

A number of simple mathematical models were devised to analyze data by Skipper, et al., (34) on repeated drug application in the treatment of L1210 leukemia in mice. Two different drugs were evaluated, first NSC 409962 (1,3-bis-(2 chloroethyl) - 1 - nitrosourea) and, secondly, amethopterin (Methotrexate). The basic model took three major factors into account, cell growth between treatment, drug retention by the host, and varying susceptibilities of cancer cells to the drug (tolerance distribution). Other factors of the models were also incorporated in variations of the basic model, such as development of a resistant strain and toxicity.

A statistical analysis of the data using a least squares criterion led to a set of "best" parameters of the model. It was learned that NSC 409962 was retained for a short time only in the host animal. Unfortunately the treatments were spaced only at one day intervals, and it was impossible to evaluate β, the drug decay parameter, precisely. It is certain that β is greater than 1 or 2, but how much greater is not known. A new set of experiments, if carried out with drug applied 2, 4 and 8 times daily, would remedy this fault. Amethopterin, by contrast, has very long drug storage. The drug gradually builds up in the system rather

than decays. A reasonable explanation lies in the fact that this drug may take quite some time to reach the site of action. Biochemical verification of this delay exists (21).

A drug resistant strain of cells was found for one set of data using NSC 409962 and was not detected in a second set of data. The reason for this, again, lies in the design of the experiments. In order to detect a resistant strain the dose and scheduling of treatment must be such as to eliminate nearly all drug-sensitive cells, since the fraction of resistant cells is normally very small and only becomes appreciable by selective survival. Only those experiments which use high doses and properly spaced treatments can identify the resistant strain of leukemic mice. These experiments should use the same dose for an increasing number of treatments, demonstrating perhaps a decreasing efficiency of the drug. Naturally this optimal regime must avoid undue toxicity to the host animal. Both resistance and toxicity, although entirely different properties work in the same direction in that both the presence of resistant leukemia cells and toxicity lead to earlier death of the host. Toxicity was not explicitly incorporated in the model; yet there was good evidence for it in the second set of NSC 409962 data set (see Chapter III, Section II-C). Here the high total-dose treatments were less effective (Figure 6) and, yet the introduction of the assumption that resistant cells appear did not improve the fit of the model. Toxicity has been demonstrated in laboratory experiments with high dose treatments of NSC 409962 (34).

An unsuccessful fit of a model to data can nevertheless lead to great insight to a problem. The amethopterin data led to an unrealistic value of β, the drug storage parameter. Using Cell Model I, β was found to be -.07, which means that more drug appears in the host than was actually injected. In order to understand the possible source of this strange result, modifications of the basic model were incorporated. The growth rate, α, had been considered constant for both treated and untreated (control) leukemic cells. A possible form of varying the growth rate was examined (see Chapter V-III-A), but led to almost no change of parameters. Another approach was to treat μ, the mean tolerance, as a decreasing function of drug concentration. This led to an absurd set of parameters and was not pursued to convergence (see Chapter V-III-B). A third attempt at finding an explanation for the unrealistic parameter β was more successful. A time delay was incorporated in the action of a drug, a variation of the basic model which led to reasonable parameters (see Chapter III-III-C). A pertinent possibly related fact is, that with respect to <u>toxicity</u> of amethopterin, there is a "more than cumulative" effect in repeated drug application (33).

In summary, it must be remembered that a model is useful only when it is simple and manageable. Population biology is a complex problem. The naive brute force approach would be to set up mathematical models reflecting, faithfully, the genetic, chemical and physiological states of the leukemic

cells and their interrelationships to the host, all constantly changing both in demographic and in evolutionary time. Clearly this would involve hundreds of parameters in equations insoluble even by high speed computers in any reasonable time. It is doubtful if the intricate results of such a parameter evaluation would have any meaning or use. We must therefore simplify the models in a way that preserves the essential features of the problem, restricting it to just a few components at a time (25).

It is this last approach that has been adopted in the present work. It is obvious that these models are oversimplified, and yet it is also probable that the consistent use of this and similar models can help unravel the kinetics of the leukemia problem. For example, this is demonstrated by the fact that a variety of future experiments is suggested by the results presented here. Presumably if these experiments were performed, new variations of these models would be needed. A combination of experimental investigations and analysis by models provides a useful procedure for subsequent studies.

APPENDIX A

RATIONALE FOR USING $Q_5 = \sum_n \log^2 \frac{10^9}{\tilde{N}_n}$

Considering any <u>one</u> of the experiments, we can drop the subscript on N_n. We thus have the following notation:

L = day of last treatment (obs.)

D = day of death (obs.)

N_k = size of population at last treatment (calc.)

$\tilde{N}$ = size of population at death (obs.) = $N_k e^{\alpha(D-L)}$

x = day of death (calc.)

Equating the calculated survivors on day of death to 10^9, we can solve for X.

$$N_k e^{(x-L)\alpha} = 10^9$$

or

$$x = \frac{9-\log_{10} N_k}{.43429\alpha} + L \qquad (1)$$

Now

$$Q_6 = \sum_n (\text{day of death (obs.)} - \text{day of death (calc.)})^2$$

where the sum is over all n experiments. Using (1), we get

$$Q_6 = \sum_n \left(D-L - \frac{9 - \log_{10} N_k}{.43429}\right)^2$$

$$= C\sum_n (D-L).43429\alpha - 9 + \log_{10} N_k)^2$$

$$\text{Where } C = \frac{1}{(.43429\alpha)^2}$$

$$= C \sum (\log_{10} N_k e^{(D-L)\alpha} - \log_{10} 10^{-9})^2$$

$$= C \sum \log^2 \frac{N_k e^{(D-L)\alpha}}{10^9} = C \sum \log^2 \frac{10^9}{\tilde{N}}$$

$$= C\, Q_5$$

APPENDIX B

EXPLORATIONS OF VARIOUS METHODS OF MINIMIZATION DESCRIBED IN CHAPTER II, SECTION III-B USING GENERATED DATA

In order to gain familiarity with minimization methods in the repeated drug application model, a set of data was generated by Monte Carlo techniques on an IBM1130 computer. The Model With Surviving Parent (Chapter 1, Section III-D) was used with parameters $\alpha = .4$, $\beta = .8$, $\mu = .47712 = \log_{10} 3$ and $\sigma^2 = 1$. The initial population size was taken to be $N_0 = 100$, and ten drug treatments of size $Z_0 = 1$ were applied at intervals of time $\gamma = 1$. The survivors obtained after each of the ten treatments were: 68, 83, 105, 135, 172, 221, 285, 367, 473, 609. The criterion function selected for this single experiment is

$$Q = \sum (\text{observed survivors} - \text{calculated survivors})^2$$

where the sum is over the ten treatments.

Starting with this random sample of ten points, an attempt was made to estimate the four parameters α, β, μ and σ^2. Most methods of estimation of four parameters are lengthy and convergence (to prespecified tolerances) slow. Convergence is obtained (although rarely) only if the first estimate is very close (see Table 25). The initial estimates used in these minimization procedures were the actual parameters used in generating the data.

In Table 24, a comparison between the two gradient methods (Fletcher-Powell and Conjugate Gradient) the Direct Search and the Linearization procedures is shown. The linearization method converged most rapidly and to the lowest Q value. This lowest Q value is in part due to more stringent convergence tolerances used in this method, i.e., 10^{-6}. For the other three methods a tolerance of 10^{-5} was used (i.e. when the change in each parameter is less than 10^{-5} the search for a minimum stops). This change in tolerances was made for economy reasons, (that is, to shorten computer time needed for convergence. The only other method that converged was the Direct Search method. The Conjugate Gradient method did not converge, even after 53 iterations. The Fletcher-Powell method was programmed to run longer and did not converge even after 153 iterations, although the least squares function Q is slightly lower than that found at convergence by the Direct Search Procedure. This can and has happened because finite tolerances are used to define convergence. What is most instructive about these results is that the minimum value of Q obtained by the Fletcher-Powell and the Direct Search method are in agreement within a relative error of 2×10^{-4}, and yet slightly different values of the parameters α, β, μ, and σ^2 are obtained. This is a characteristic difficulty in optimization procedures with several parameters. Often the parameters are inaccurately determined by minimization procedures which lead to a value of the function which is very close to the minimum, but not exactly equal to the minimum.

TABLE 24

COMPARISONS OF MINIMIZATION METHODS DESCRIBED IN CHAPTER II, SECTION III-B ON DATA GENERATED WITH MODEL WITH SURVIVING PARENT PARAMETERS LISTED IN APPENDIX B *

Method	Final Parameters				Final Q
	α	β	μ	σ^2	
Linearization	.399575	.820135	.478162	1.048490	.169364
	(Converged after 4 iterations. Very sensitive to choice of first estimate)				
Gradient Methods					
Fletcher-Powell	.399832	.806282	.486132	1.088725	.17159
	(No convergence after 135 iterations)				
Conjugate-Gradient	.401805	.79986	.471278	1.001169	.2387
	(No convergence after 53 iterations)				
Direct Search	.399986	.815109	.477103	1.038704	.17163
	(Less sensitive than linearization method to choice of first estimate)				

*Initial estimate for the four parameters in all the methods were the parameters used in generating the data. See text.

In Table 25, the fact that the optimization procedures do not necessarily lead to a minimum least squares value. In this work two parameters were kept fixed ($\alpha = .4, \beta = .8$) and Q was minimized as a function of μ and σ^2 only. Actually in the case of just two unknown parameters, the choice of a good starting point is not as critical as in the case of three of more unknown parameters. Nonetheless, Table 24 shows that 1) convergence is much more rapid if the starting point is close to the true value of the parameters, 2) convergence to the true absolute minimum Q cannot be assured if the starting point is far from the true values of the parameters. What is noticeable in addition is that the lowest value of Q is obtained on line 2 of Table 25. This differs by more than one part per thousand from the results shown in lines 1, 3, 4 and 5, which are essentially identical. It is not clear whether the result of line 2 is a different local minimum or whether the function Q_5 is so flat nearby that the tolerance requirements for convergence are satisfied before the minimum is reached.

TABLE 25

COMPARISON OF DIFFERENT STARTING POINTS AND OUTCOME IN DIRECT SEARCH MINIMIZATION METHOD USING GENERATED DATA
α IS FIXED AT 0.4, β IS FIXED AT 0.8

Init. Guess μ	Init. Guess σ^2	Init. Q	Final Value μ	Final Value σ^2	Final Q	Number of Iterations
.3	.8	$.666 \times 10^5$	.483745	1.100008	.173536	89 *
.3	1.2	$.775 \times 10^5$	.489014	1.102255	.173299	135 *
.2	.8	$.157 \times 10^6$	.488746	1.100034	.173532	210 *
.4	1.	$.157 \times 10^5$	.488745	1.100008	.173536	90 *
.47712	1.	$.167 \times 10^1$	.488821	1.100644	.173433	80 *
.4	.8	$.865 \times 10^4$	.444537	.76069	7.11910	840

* Converged

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