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TERMINAL PROGRESS REPORT

U.S. AEC CONTRACT NO. AT-(40-1)-3884

A RADIOBIOLOGIC EVALUATION OF AN IN VITRO  
MAMMALIAN CELL RENEWAL SYSTEM

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September 25, 1972

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PROGRESS REPORT

US AEC CONTRACT NO. AT-(40-1)-3884

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1. This progress report covers the period 1 Jan 69 to 1 Aug 69.
2. The Chinese Hamster (CHO) cells were kindly provided by Dr. Hahn of Stanford University. In earlier work Hahn and his colleagues showed that the transition from log growth to plateau phase produced rather striking changes in the cell's radiobiologic properties (1, 2). Figure 1 from our laboratory shows an example. The upper curve shows the survival of cells in log growth. Notice the prominent shoulder; the extrapolation number is 2.75. The lower curve shows the response of the plateau phase cells. Now, the shoulder is virtually absent; the extrapolation number has dropped to 1.0. The slope though, seems relatively unchanged as compared with the log phase cells. Paired dose experiments indicate that the usual "repair" segment becomes markedly reduced in plateau phase--as compared to log phase.

As a possible explanation, Stewart, et al, have proposed the following. They suggest that perhaps during the transition from log growth to plateau phase, the cells assume a "resting stage" (2). These authors, however, do not suggest actual mechanism to explain the behaviour of plateau cells. We believe that some recent biochemical data applies to this problem.

The work of Okazaki with bacteria indicates that DNA is synthesized in a quantal manner (3). The interpretation of these results suggests the following: Discrete portions of the DNA molecule are replicated (by

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HELIOS PROGRAM PROGRESS REPORT

15 September 1972

by

Harry S. Robertson

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Helios Program Progress Report

27 August 1971

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## INTRODUCTION

Cell renewal systems represent the basis of life in man. A continuous process of formation and destruction of such diverse elements as blood cells, germ cells, epithelium, etc., must be balanced in order to maintain life. One of the most profound effects of ionizing radiation is to alter cell renewal systems such that the formation of new cellular elements stops while the destructive processes continue. The ultimate result is a loss (or at least a severe impairment) of the function of the tissue involved.

Two broad experimental tools have been available to those wanting to study the nature of mammalian cell renewal systems. The first is the intact animal. While valuable work certainly has been done with animals, many observed effects are potentially confounded by simultaneously occurring alterations of other aspects of the animal's physiology. A second tool is the cultured mammalian cell. While these cells allow effects to be observed at the cellular level without the influence of uncontrolled exogenous factors, the cell population is essentially artificial.

However, where cell renewal systems, *in vivo*, are characterized by a constant number of cells, the cultured cells are usually studied while in exponential growth. Exponentially growing cells are often used by radiobiologists as model systems to study the radiation response of human neoplasms and to attempt to predict and explain their behavior *in vivo*.

As our knowledge of cell kinetics has increased, it has become apparent that the simple culture system is inadequate. In many aspects,

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exponentially growing cells do not match those of tumors in kinetics of growth. An example of this is an analysis by Mendelsohn of the proliferation characteristics of a mammary tumor in the C3H mouse (1). He demonstrated that only a small portion of a tumor cell population will exhibit a repeating pattern of cell cycle phases. Perhaps 60-80% of the cells are resting. This was shown by their inability to incorporate tritiated thymidine ( $^3\text{HTdR}$ ) even after continuous labeling for several intermitotic periods. In marked contrast, cells in exponential growth approach 100% labeling after an exposure time of about one mean generation time. If the growth fraction (ratio of cycling cells to total cells) is unity, then all the cells are cycling. If it can be shown that resting cells can be induced into the cycle, given the proper stimulus, then the magnitude of the growth fraction and the radiation response of the non-cycling cells can be very important in determining the tumor's response to therapy. However, the possibility of a nonproliferative compartment seems remote in exponentially growing cells. Experimenting on a system having a growth fraction of one may yield results with limited relevance when applied to a system having a much lower growth fraction.

Another characteristic of tumor cell populations is that cells die and disappear from the population without giving rise to viable progeny (2). However, in many established cell lines the cycle of one cell giving rise to two more cells is repeated in all cells, with few exceptions. In a line of Rat Sarcoma cells (RT-2) used in our laboratory growth kinetics data indicate a cell turnover rate of less than 2% per day in an exponentially growing population. Watanabe and Okada, using

a mouse lymphoma cell line (L5178Y) and an eosin staining technique to estimate the fraction of dead and/or dying cells, report a cell loss of less than 6% for exponentially growing cells (3). Hahn, using a line of Chinese Hamster (HA-2), reports that DNA turnover data indicate less than 1% of the exponentially growing population dies during a 24 hour period (4). These are but a few examples.

Recently Hahn described a means of developing a steady-state population of cultured cells which has many of the properties of a tumor or a cell renewal system (5). He found that after seeding monolayer bottles, Chinese Hamster cells increase exponentially in number for some 4-5 days. After this, however, the number levels off and reaches a stable value. The population remains stable even if the medium is replenished every few hours. This seems to exclude anoxia or starvation as an important factor. At the time of the steady state, he found about 10% of his cells to be lost from the monolayer per day. Since these lost elements are rapidly replaced by new cells, the population size will stay constant. Consequently, a cell renewal system, with a daily turnover fraction of 0.1 was produced.

Since Hahn's initial report (5), several workers have reported similar findings using several different cell lines. For example: Watanabe, et al., using a mouse lymphoma line (L5178Y)(3); Chapman, et al., using a Chinese Hamster line (V79-376A)(6); Berry, et al., using a Chinese Hamster line (CHL-F)(7); Nash, et al., using a Rat Sarcoma line (RT-2)(8); Madoc-Jones, et al., using an L cell line (L60)(9); and Little, using Chang liver cells (10).

Hahn performed a few pilot radiobiologic studies with this system which have considerable importance. He found the slope of the dose response curve of the steady state cells (these will be called "plateau" phase cells) to be equal to the slope of the curve for exponentially growing cells. The extrapolation number, where above 2 for exponentially growing cells, had fallen to 1 for plateau phase cells. Hahn correctly surmised the reduction in the extrapolation number to mean a decreased ability to repair sub-lethal injury, as a paired-dose experiment showed. Also, his initial experience, albeit incomplete, suggests the extrapolation number to rise when the cells began to grow exponentially again.

For several years radiobiologists have sought a method for producing cultured mammalian cells which either possess or which lack the ability to repair sub-lethal injury. Hahn's system seems, at this point, to represent a means of producing mammalian cells, in quantity, which have these characteristics. A primary goal of our research, then, has concerned the radiobiologic properties of plateau phase, as contrasted to cells in logarithmic growth.

## METHODS

### 1. Cell Survival Techniques

The methods used in our laboratory are already in print (11, 12). Briefly, the Chinese Hamster cells (CHO) and Rat Sarcoma cells (RT-2) are carried in monolayers until use. For radiation experiments, the cells are trypsinized (or scraped), diluted, plated, allowed a period of time to attach, irradiated and/or treated with other agents, and incubated for 1-2

weeks to allow colony growth. The cells are kept in a water jacketed incubator at 37°C in a high humidity, 95% air 5% CO<sub>2</sub>, atmosphere.

## 2. Polynucleotide Kinase

We have previously described the use of an enzyme, polynucleotide kinase, to measure the kinetics of rejoining of post-irradiation DNA breaks characterized by 5' termini (13, 14). The methods used for this assay are already in print. This enzyme is extracted from E. coli which has been infected with T-2 phage (15).

Briefly, the cultured cells are brought into suspension, irradiated and then lysed with sodium dodecyl sulfate (SDS). After lysis, the cells are treated with boiled RNase, pronase, chloroform-isoamyl alcohol (24:1), and then dialyzed against tris buffer. Following dialysis, the DNA is treated with E. coli alkaline phosphatase (to convert 5'PO<sub>4</sub> termini to 5'OH termini) and then treated with polynucleotide kinase and  $\gamma$ -labeled AT<sup>32</sup>P (16). The radioactivity, then, is rendered acid insoluble, and, consequently, the DNA specific activity is proportional to the number of 5' termini within the DNA molecule.

## 3. Ultracentrifuge Method

We use the method described by Humphrey, et al., with minimal modification (17). The cells are pre-labeled with <sup>3</sup>HTdR or <sup>14</sup>CTdR, suspended, irradiated, and then lysed with a solution of 2% tri-iso-propyl naphthalene sulfonic acid (TIPNS), 1% p-aminosalicylate, and 6% sec-butyl alcohol adjusted to pH 12.5 with NaOH. A 50 - 100 $\lambda$  portion of the lysate (contains about 10  $\mu$ g DNA) is layered onto a 5% - 20% alkaline sucrose gradient (18), and then centrifuged at 30,000 rpm for 3 hours in a fixed

angle rotor (Type 40 - Spinco). After centrifugation, 7 drop fractions are collected into counting vials, counting fluid added and the vials counted.

#### 4. Biochemical Methods

The rate of synthesis of DNA, RNA, and protein is determined by measuring the rate of incorporation of  $^3\text{HTdR}$ ,  $^3\text{HUR}$ , and  $^3\text{H}$  leucine into the DNA, RNA, and protein components of the cell acid insoluble fraction. The acid insoluble fraction is resolved into these components by a modified Schmidt-Thannhauser method (19). The DNA content is measured by the diphenylamine method (20), the RNA content by the orcinol method (21), and the protein content by the Lowry method (22). The ATP content of the acid soluble fraction is measured by the firefly luciferase method (1).

#### 5. Irradiation Methods

The cells were irradiated with a Westinghouse 250 kVp X-ray unit. The beam was filtered to yield a HVL of 1 mm Cu. The dose rate was 100 rads/min.

#### 6. Autoradiographic Methods

The procedure used in our laboratory for autoradiography is a modification of that described by Joffes (23). Briefly, the procedure used is as follows. After the appropriate labeling and handling, the surface containing the cells is coated with Kodak NTB-3 nuclear track emulsion (diluted 1:1 with distilled water). After an appropriate exposure time (3-6 weeks for RT-2 cells) the preparations are developed in Kodak D-19 developer and fixed in Kodak acid fixer. The slides are then washed and stained with Giemsa stain. After drying, the cells were scored.

## RESULTS AND DISCUSSION

We measured a number of growth curves for the CHO cells. We found that by the 4th day after seeding, exponential growth had ceased and the cells entered plateau phase. Consequently, we used cultures of 4 days or older for experiments with plateau phase cells. At this point we frequently experienced difficulty because of sloughing of cells from the monolayer. The results for CHO cells which follow were provided by monolayers which remained attached. Because of this sloughing problem, a portion of the experimentation utilized CHO cells in plateau phase, while other studies used another cell type (RT-2). The report will be subdivided according to cell type.

A. Experiments with Chinese Hamster Cells

Figure 1 shows a survival curve for CHO cells in plateau and log growth phases. The shape of the survival curve for the log phase cells is typical for low L. E. T. radiations. There is a definite shoulder and a well defined log-linear segment. The plateau phase cells, however, showed virtually no shoulder; the  $D_0$  values were essentially the same for the two groups of cells.

Hahn also performed paired-dose studies (5). He found the typical "repair" pattern to be markedly reduced for plateau phase cells. From results such as these, then, plateau phase cells would seem to be less able to repair sub-lethal radiation injury than cells in log growth.

Table 1 contains the results of biochemical measurements which contrast the rate of synthesis of DNA, RNA, and protein; the ATP contents are also included. For these studies, monolayers of CHO plateau and log phase cells were used. The old medium was removed and replaced with fresh

medium which contained either  $^3\text{HTdR}$ ,  $^3\text{HUR}$ , or  $^3\text{H}$  leucine (1.0  $\mu\text{Ci/ml}$ ). After a 30-minute incubation, the cells were detached with a rubber policeman and a portion taken for cell counts (these cells were treated with 0.02% trypsin to break up clumps). The cell suspension was treated with perchloric acid (final concentration 0.5N) and the acid insoluble fraction resolved into the DNA, RNA, and protein components as indicated in the Methods section.

TABLE I

## Experimental Results - CHO Cells

<u>Compound</u>	<u>Log Phase</u>	<u>Plateau Phase</u>	<u>Log/Plateau Ratio</u>
DNA Specific * Activity ( $^3\text{HTdR}$ Label)	2261	31.5	71.8
RNA Specific * Activity ( $^3\text{HUR}$ Label)	2533	51.5	49.2
Protein Specific * Activity ( $^3\text{H}$ leucine label)	32.	42.	0.76
ATP Content †	214	95	2.25
* cpm/ $\mu\text{g}$			
† picomoles/ $10^6$ cells			

These results indicate that CHO plateau phase cells have a considerably lower rate of incorporation of DNA and RNA precursors, as compared to log phase cells. Also, the ATP content of plateau phase cells is considerably reduced. Although these results would suggest the rates of synthesis of DNA and RNA to be reduced, we are hesitant to

make this statement on the basis of these results alone, because we cannot exclude the possibility that the rate of transport of the precursors could be depressed while the rate of synthesis remained normal. Since the rate of incorporation of  $^3\text{H}$  leucine into protein was similar for the two groups, we favor the notion that the rates of DNA and RNA synthesis are depressed.

To investigate this point further we made autoradiograms of cells pre-labeled with  $^3\text{HTdR}$ . The results are contained in Table II.

TABLE II

## Autoradiographic Results

<u>Phase</u>	<u>Per Cent Labeled Cells</u>
Log	62.8
Plateau	7.6

Results such as these, then, indicate that the cells are not in S phase. These findings would agree with Hahn, et al., who suggested that the cells are in  $G_1$  (5).

Because the cellular DNA represents a radiobiologic "target" of such importance, we performed a series of experiments in which the rejoining of DNA breaks was measured by the polynucleotide kinase method.

Figure 2 shows the effect of 1000 rads of 250 kVp X-rays on CHO cells in log growth. The cells, shown in the left panel, were suspended in glucose free Hank's balanced salts solution while the cells of the right panel were suspended in  $10^{-4}\text{M}$  2,4-dinitrophenol (DNP); the

DNP was dissolved in HBSS. Immediately after irradiation, the largest number of DNA breaks (characterized by 5' termini) were present. These breaks, however, were rapidly rejoined--as indicated by a decreased number of 5' termini. Figure 3 shows the effect of 1000 rads of 250 kVp X-rays on plateau phase Chinese Hamster cells. As before, the cells were detached from the monolayers and suspended in glucose free Hank's balanced salts solution (HBSS) or in  $10^{-4}M$  DNP. Immediately after irradiation, the largest number of DNA breaks (characterized by 5' termini) were present. With time after irradiation, the number of breaks decreased (presumably due to rejoining). The rate of rejoining, though, was slower than for other lines of cells in log growth. In other experiments we have found the control (non-irradiated) DNA of the plateau phase cells to contain more 5' termini than control DNA from log phase cells. The differences in the control DNA specific activities shown in Figures 2 and 3 are a result of differences in the  $AT^{32}P$  specific activities used for the polynucleotide kinase assay.

Figure 4 shows the effect of 10,000 rads on plateau phase CHO cells as measured by alkaline sucrose sedimentation. These cells were pre-labeled with  $^3H$ TdR for 3 days before the experiment. The cells were detached from the monolayers with trypsin, washed with HBSS and then suspended in HBSS at a concentration of  $10^6$  cells/ml. A control sample was removed and the cells then irradiated. After exposure samples were collected and lysed, as described in the Methods section.

As the figure shows, the DNA of the control cells sedimented more rapidly than the DNA from the irradiated cells. Also, there is minimal evidence of rejoining of the DNA breaks for cells lysed 60 minutes

after irradiation, as compared with cells lysed immediately after exposure. Log phase cells, on the other hand, show evidence of DNA rejoining as early as 5 minutes after irradiation. Results such as these, then, agree with the polynucleotide kinase data. Namely, plateau phase cells rejoin DNA breaks slower than cells in log growth.

As a possible explanation of the behaviour of plateau phase cells, Stewart, et al., have suggested that cells assume a "resting stage" while passing from log phase to plateau phase (24). The studies of Okazaki with microorganisms suggest that DNA is synthesized in a discrete (or "quantal") manner (25). Consequently, DNA would be synthesized in small segments by DNA polymerase and the segments would be joined by an enzyme such as DNA ligase. Perhaps, when the cells move from log to plateau phase they become deficient in DNA ligase (or if the endogenous components necessary for ligase action were deficient). The DNA then, would be partially synthesized by DNA polymerase, but many single strand breaks would exist because of the failure of DNA ligase to join the smaller units. The increased number of endogenous single strand DNA breaks would render a given dose of radiation more effective because the probability of producing double strand (and lethal) DNA breaks would be increased. Also, the capacity for retention of sub-lethal radiation injury (as indicated by the size of the shoulder of the survival curve) would be reduced.

An interesting difference between plateau and log phase cells has been described by Mego, et al., (26). These workers, using alkaline sucrose gradients, examined the sedimentation profiles of DNA from Chinese Hamster cells. The data they obtained indicated that under

experimental conditions the DNA of exponentially growing cells banded in a narrow distribution near the end of the gradient tube, while DNA from plateau phase cells banded in a somewhat wider distribution near the beginning of the gradient. These authors also report that plateau phase cells that were induced to obtain exponential growth (by subculturing) and measured in exponential growth do not exhibit such a sedimentation pattern, but appear exactly as does DNA from normal exponentially growing cells. This indicates that the diffuse sedimentation pattern of DNA from stationary phase cells is a function of the macromolecular effects (whatever) of the plateau-phase itself. Schandl and Taylor using neutral sucrose gradients report no such sedimentation pattern shift for double stranded DNA (27). In sucrose gradients, travel along the gradient is a function of the size of the molecule. In alkaline sucrose gradients, one is looking at single stranded DNA, and in neutral gradients one sees double stranded DNA. This sedimentation shift seen with single stranded DNA and lack of same with double stranded DNA is indicative of nonadjacent single strand breaks (or nicks) in alternate strands of the molecule. The presence of a large number of single strand breaks in the DNA of plateau phase cells would be consistent with the reduced rate of rejoining of such breaks as shown by our polynucleotide kinase and ultracentrifuge data, the reduced (or absent) shoulder on the X-ray survival curve, and the cells impaired ability to repair sublethal injury.

Hahn offers more evidence consistent with the hypothesis that DNA of non-nutrient deficient plateau phase cells contains a large number

of endogenous single strand breaks (4). He performed experiments with the monofunctional alkylating agent methyl methanesulfonate (28). This drug's primary action on DNA is the methylation of guanine. After depurination and hydrolysis, this initial lesion may lead to the induction of single-strand breaks (29). Hahn reports that a survival curve of Chinese Hamster cells in exponential growth and exposed to graded doses of the alkylating agent had a wide shoulder with an extrapolation number of nearly 100. The shoulder disappeared completely if plateau-phase cells were similarly exposed. An explanation consistent with this finding would be the presence of a larger number of single strand breaks in the DNA of plateau cells.

B. Experiments with Rat Sarcoma Cells (RT-2)

In Hahn's initial description of a method to produce a steady-state population of cultured cells, he established the presence of *in vitro* cell renewal systems (5). The study of the radiation response of cell renewal systems could have great implication, especially in the area of radiotherapy. Since radiotherapy is the treatment choice for many types of cancer, it is important then, that we know as much as possible about the radiation response of not only normal tissue, but also neoplastic tissue. There has been a great deal of work done on the radiation response of mammalian tissue. Unfortunately, the majority of the *in vitro* investigation has been accomplished using relatively radiosensitive, non-neoplastic tissue. In short, normal tissue. Several of the cell lines used are of neoplastic origin, but very few of these have retained to any extent their malignant qualities. The response of these tissues would not truly represent that of neoplastic tissue.

To study the response of neoplastic tissue, an ideal choice would be a cell line of neoplastic origin that retains to some degree, preferably high, its malignant qualities. The cell line should lend itself to common tissue culture techniques with ease, and it would help considerably if there were a common human counterpart observed clinically.

Just such a cell line is available for use. This cell line has been designated Rat Sarcoma, RT-2 (30). The cell line originated from a primary fibrosarcoma found in a Fischer rat. It is readily adaptable to tissue culture, and it retains to a great degree its malignant qualities. The cell line is readily transferred back and forth from animal (Fischer rat) to culture vessel (Monolayer). Because of (1) its histologic and morphologic similarity to many human fibrosarcomas, (2) the ease with which it can be handled in culture (this includes not only exponential growth but plateau as well), and (3) the retention of its malignant qualities, the RT-2 cell line would be an ideal tool not only for the study of the radiation response of neoplastic tissue, but for cell renewal systems also.

The RT-2 cell line in our laboratory originated from a primary sarcoma in a Fischer rat. The primary sarcoma was a result of the infection of the rat by the ova of Taenia taeniaformis (30). The tumor, *in vivo*, is a very slow growing fibrosarcoma with considerable dense fibrous stroma. A Fischer rat exhibiting a well-developed tumor is shown in Figure 5-A. The gross tumor is shown in Plate B and a histologic section of such a tumor is shown in Plate C. Cultured RT-2 cells as a monolayer are shown in Plate D. The cell line grows very well in culture

and forms well-defined, compact colonies as shown in Figure 6. These petri plates contain colonies derived from cells exposed to graded doses of X-rays: from 0 rads at lower right to 1000 rads at upper left. The cultured cells exhibit a fair degree of polymorphism and retain to a high degree their malignant qualities (30).

The Rat Sarcoma (RT-2) cells are carried as monolayers in plastic culture flasks. They are nourished with Eagle's minimum essential medium (MEM) supplemented with calf serum. The MEM is supplemented with 10% calf serum for cells carried as monolayers and 20% calf serum for single cell experiments. The monolayers are maintained at 37°C. with air as the gas phase. New monolayers are routinely initiated every 7 days. A monolayer from a large culture flask is trypsinized, or scraped, washed with Hank's balanced salt solution (HBSS) and resuspended in fresh medium. This cell suspension usually contains from 7 to 10 million cells. The suspension is then divided among 5 flasks containing MEM supplemented with 10% calf serum. For normal maintenance the medium is changed on the fifth day after seeding. Only if the cells are to be maintained as monolayers for a longer time is the medium changed more often.

The cells in culture attain logarithmic growth in about 24 hours after seeding. Monolayers of cells in logarithmic growth will usually attain a plateau stage 4 - 6 days after seeding (Figure 7). At this time there is a minimum daily turnover of cells. The time the monolayer attains plateau is determined by the number of cells initially seeded in the culture flask. Plateau phase monolayers may be maintained for several

days (up to 20) provided the medium is changed daily during this time period.

In single cell survival experiments, the procedures outlined by Puck and Marcus (31) were used. The monolayers are harvested by trypsinization or scraping at least three days after seeding (3 days for log phase, 6-7 days for plateau). The cells are washed in fresh medium by centrifugation and resuspended in 5 ml of medium. The cell density is determined by counting a portion of this suspension with a hemocytometer. The volume of the cell suspension is adjusted to contain a suspension of  $10^6$  cells/ml. From this suspension further dilutions are made as required. The desired number of cells are then seeded into 60 mm plastic petri dishes containing 5 ml of medium. In most cases the cells are allowed to attach for 24 hours prior to treatment. Plateau-stage cells are treated immediately after plating. After treatment, the petri plates are maintained at 37°C. in a high humidity, 95% air, 5% CO<sub>2</sub> atmosphere for 9 - 14 days. At the end of this period, the plates are fixed with 10% formalin, stained with methylene blue, and the colonies counted.

In preparation for certain labeling experiments monolayers are trypsinized, washed with HBSS, and resuspended in either glucose free HBSS or fresh media. The cell density is determined by counting as before. Aliquots of this suspension are used as required for various labeling experiments.

#### GENERATION CYCLE ANALYSIS

The *in vitro* generation cycle analysis of the RT-2 cell line is accomplished using autoradiographic, clone size analysis, and cell counting techniques. The parameters in question are:

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- I. The time of the cell cycle,  $T_C$ ;
- II. The size of the proliferative pool;
- III. The times of the phases of the cell cycle;
  - a. The time the cell is in mitosis,  $T_M$ ;
  - b. The time of the immediate post-mitotic, pre-synthetic (DNA) phase,  $T_{G1}$ ;
  - c. The time of DNA synthesis,  $T_S$ ;
  - d. The time of the post-DNA synthesis phase,  $T_{G2}$ .

Approximately  $5 \times 10^4$  cells were seeded into plastic culture flasks (25 cm<sup>2</sup>, 30 ml size) containing MEM supplemented with 10% calf serum. Forty-eight hours after seeding, <sup>3</sup>H-thymidine (5 uci/ml, 20.2 Ci/mM thymidine) was added to each of the flasks. Each hour for 20 hours after addition of the label, the medium was removed from replicate flasks. The flasks were rinsed with Hank's balanced salts solution (HBSS) and the cells fixed with 10% formalin. After fixing, the flasks were washed with HBSS until no radioactivity above background appeared in the wash. The top and sides of the flasks were removed forming a plastic slide. Kodak nuclear track emulsion type NTB-3 was then (diluted 1:1 with distilled water) applied over the cells. After exposure for 35 to 45 days at 4°C., the slides were developed in Kodak D-19 for 4 minutes at 21°C. and fixed in Kodak Acid Fixer for 3 minutes. The slides were washed in water at 24°C. for one hour and then stained with Giemsa stain in phosphate buffer.

In certain experiments, a pulse label of 15 minutes was used in place of continuous labeling. Otherwise, all the flasks were handled in the same manner. The cell cycle time,  $T_C$ , was determined using a pulse label after the method of Quastler and Sherman (32).

After microscopic examination, and scoring, the Labeling index, the mitotic index, and the % labeled mitotic figures were determined. From this information, estimates of the parameters characterizing the cell line can be determined.

Figure 8 shows the results of exposing the RT-2 cells to a continuous label of  $^3\text{HTdR}$ . The labeling index is plotted as a function of time of exposure to  $^3\text{HTdR}$ , and reaches 100% in about 13 hours. The fact that it reaches 100% indicates a growth fraction of 1.00. That is, all the cells are participating in the cell cycle. The y intercept value of about 28% is a measure of the proportion of cells in s phase at any one time. The time required for the labeling index to reach 100% is a measure of the time of  $G_2 + M + G_1$ : 13 hours (33).

An estimate of the time of  $G_2$  was obtained by exposing cells in plastic flasks to a pulse label of  $^3\text{HTdR}$ . The results of such an experiment are shown in Figure 9. The extrapolation, to the abscissa, of the first ascending wave of labeled mitoses to the X axis gives an estimate of the time of  $G_2$  (32). This is 1.54 hours. The cell cycle time can also be measured by this procedure, using the time interval between the 50% intercepts of the first and second ascending waves of labeled mitoses (32). The cell cycle time as determined by this procedure is 21.0 hours.

The cell cycle time was also determined by two other methods. One is a clone size analysis. A known number of cells (approximately  $10^4$ ) are seeded into plastic petri plates containing MEM supplemented with 20% calf serum. The plates are maintained under standard growth conditions for 20 hours. At this time, duplicate plates are removed and

fixed and stained each hour thereafter. After drying, the plates are examined microscopically and the number of cells/colony determined. This value is plotted on semi-logarithmic paper as a function of time after the initial 20 hours. The doubling time is determined from the log-linear portion of the growth curve. The cell cycle time as determined in this manner was 21.0 hours (Figure 10).

The third method involved determining the total number of cells as a function of time after seeding into culture flasks. A known number of cells ( $10^4$  to  $10^5$ ) are seeded into several plastic culture flasks containing MEM supplemented with 20% calf serum. The flasks are sealed and maintained at  $37^\circ\text{C}$ . Every 24 hours after seeding, duplicate flasks are removed. Each flask is thoroughly trypsinized with 0.2% trypsin in HBSS and all the cells removed. The cells are washed by centrifugation and resuspended in 2 ml of fresh MEM. The cell density is determined by multiple counts on a hemocytometer. The number of cells/flask is plotted as a function of time after seeding. The cell cycle time is determined from the log-linear portion of the resulting growth curve, and is 21.5 hours (Figure 7).

As mentioned above, the time of  $G_2 + G_1 + M$  was estimated to be 13 hours. The cell cycle time is equal to the time of  $(G_2 + G_1 + M) + S$  and is 21 hours in length. It follows that the time of  $S$  is equal to 21 minus 13 hours or 8.00 hours.

The duration time of mitosis was determined by calculation using a method after Stanners and Till (34). In this calculation, the time of mitosis ( $T_M$ ) is equal to the product of the mitotic index ( $M$ ) and the time of the cell cycle ( $T_C$ ), divided by the base  $e$  logarithm of two (.693) (Eq. 1).

Eq. 1 
$$T_M = \frac{M \times T_C}{.693}$$

The mitotic index of cells in exponential growth is 0.0263 and the cell cycle time ( $T_C$ ) 21 hours. From this calculation  $T_M$  is equal to 0.8 hours.

The time of  $G_1$  is obtained by subtracting the sum of ( $T_{G2} + T_S + T_M$ ) from the cell cycle time (21 hours) and is equal to 10.66 hours. These data from the cell cycle analysis are summarized in Table III.

TABLE III

Experimental Results RT-2 Cells

$T_M$	=	0.8 hours
$T_{G1}$	=	10.66 hours
$T_S$	=	8.00 hours
$T_{G2}$	=	1.54 hours
<hr/>		
$T_C$	=	21.0 hours
<hr/>		

Monolayers of cells are used for biochemical studies. The studies were performed using cells in both plateau and logarithmic growth.

Tritiated precursors to DNA, RNA, and protein were used for isotopic measurement;  $^3H$ -thymidine (20.2 ci/mM) was used as a label for DNA.  $^3H$ -uridine (24.9 ci/mM) was used for RNA, and  $^3H$ -leucine (57.6 ci/mM) for protein. The labeling times varied from 15 to 30 minutes in most cases and for 72 hours in a few. The labeling was done in HBSS or MEM supplemented with calf serum at 1 uci/ml, 2.5 uci/ml, or 5 uci/ml. All

radioactivity determinations were made using a Beckman Liquid Scintillation Counter, Model LSC-200. The counting solution was toluene with diphenyl oxazole as the scintillator. Beckman Biosolv, BBS-3, was used as a solubilizing agent.

In the studies on log-plateau comparisons, bottles of log-phase and plateau-phase cells were used as monolayers. The medium from each flask was removed and replaced with fresh glucose free HBSS/fresh MEM containing any compounds being tested. The flasks were incubated at 37°C. for the desired time after addition of the labeled compounds. After the labeling period, the monolayers were trypsinized or scraped with a rubber policeman and washed, by centrifugation, with glucose free HBSS. The cells were then resuspended in fresh glucose free HBSS. In other studies, the cells from several monolayers were trypsinized and washed, by centrifugation, with glucose free HBSS. The cells were then resuspended in fresh glucose free HBSS. If necessary, cell counts were made at this time with a hemocytometer. Portions of this suspension were then added to flasks containing an HBSS solution of any compound being studied. The flasks were incubated at 37°C. and at the proper time, the cells were pulse labeled with the appropriate labeled compound. Following the labeling period, the cell suspension was treated with perchloric acid (final concentration of 0.5N) and the acid insoluble fraction resolved into the DNA, RNA, and protein components as indicated in the methods section.

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TABLE IV

Experimental Results RT-2 Cells

<u>Compound</u>	<u>Log Phase</u>	<u>Plateau Phase</u>		<u>Log Plateau Day 9 Ratio</u>
		<u>9 Day</u>	<u>13 Day</u>	
DNA Specific * Activity ( <sup>3</sup> Htdr label)	1008.5	168.7	125.3	5.97
RNA Specific * Activity ( <sup>3</sup> HUR Label)	71.4	32.3	23.0	2.21
Protein Specific * Activity ( <sup>3</sup> H-Leucine Label)	14.1	17.3	15.4	.815

\*cpm/ $\mu$ g

As with the Chinese Hamster plateau-phase cells, these results indicate that RT-2 plateau phase cells have a much lower rate of incorporation of DNA and RNA precursors, as compared to cells in exponential growth. It seems as if incorporation of protein precursors is independent of culture age. This would indicate a high rate of protein turnover. Autoradiographic data from similar experiments show that while the proportion of the population of cells synthesizing DNA in log phase is greater than 25-30%, it is only about 1-3% for cells in plateau phase. Additional evidence for a depressed rate of synthesis of DNA (and probably RNA) is shown in Figure 11. These data are derived from autoradiographic experiments in which both log and plateau phase cells are exposed to a continuous label of <sup>3</sup>HTdR. After the appropriate preparation, the labeling index was determined as a function of time after addition of the label. Note the difference in slope of the two curves. The slope

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of the upper curve (log phase cells) should be indicative of the rate of incorporation of <sup>3</sup>H-TdR into the nucleus of log phase cells since only labeled nuclei were scored. The lower curve, plateau phase cells, has a much smaller slope. It should be noted that the upper curve is very similar to that shown in Figure 8. (The curve in Figure 8 was derived by scoring of labeled cells, not just labeled nuclei.) For the time duration indicated (Figure 11), the lower curve did not reach 100% labeling. However, after a period of approximately 72 hours the plateau cells did approach 100% labeling (91%). This would indicate a growth fraction of just less than unity and a greatly elongated cell cycle. The decreased growth fraction is probably due to loss of viability, but not attachment, by a small portion of the population. There appears to be about a 10% renewal of the population per day. Those cells, then, could account for these results.

In summary, the plateau phase RT-2 cells are definitely different than their log phase counterpart in respect to their growth and macromolecular synthesis characteristics. These data, with the exception of the level of RNA synthesis, are compatible with those of Watanabe, et al.; Hahn; and Levine, et al. (3, 4, 35). Hahn (4) indicates a level of RNA synthesis for plateau-phase cells that is independent of culture age. We did not find this in either the CHO cell line in our laboratory or in the RT-2 cell line.

Surprisingly, in view of the large amount of data available on cells in exponential growth, reports on the radiation response of plateau-phase cells are few. Modoc-Jones found a marked increase in the extrapolation

number ( $n$ ) as rat sarcoma cells went into the plateau phase, while the slope of the survival curve became steeper (decrease in  $D_0$ ) (36). Ludovici, et al. found that survival curve parameters of irradiated HeLa cells depended only weakly on culture age or on cell density (37). This finding is also reported by Glivos and North (38).

A comparison of the response of both the plateau and log phase RT-2 cells to a single exposure of 250 kVp X-rays is shown in Figure 13. The cells were prepared as described above. The plateau phase cells were irradiated immediately after plating. The cells in both stages of growth are resistant to radiation damage. These data are qualitatively like those of Watanabe, et al., and Hahn in that the  $D_0$ 's of the two stages are similar while the extrapolation numbers are different (3, 4, 5). The lowered extrapolation number in the plateau phase indicates an impaired ability to repair or modify sublethal radiation damage. Hahn reports a complete loss of shoulder in his Chinese Hamster plateau cells and thus a lack of the ability to modify sublethal radiation injury (5). Although our RT-2 cells can still modify sublethal injury (as indicated by the small shoulder) we feel these data are compatible with those of Hahn. An explanation for the presence of our small shoulder could lie in the different method of handling our plateau phase cells. Hahn (and others) irradiates his plateau-phase cells in the petri dish in which they were grown, before any perturbation such as trypsinizing or scraping. We disturbed the monolayer by trypsinization, dilution (this took about 15 minutes), and plating prior to irradiation. This short interval may be just long enough for the cells to regain some

of their log-phase characteristics. Regardless of the differences, the two phases are still quite radio-resistant, similar to many *in vivo* fibrosarcomas.

It should be noted that Little, using a human cell line (Chang) reports that after irradiation in plateau-phase, not only do these human cells recover from sublethal radiation damage, but potentially lethal damage is repaired if the cells are allowed to remain in the stationary phase for some time after irradiation (10). Also interesting is that Hahn, in measuring the ability to modify sublethal injury of nutrient-deficient plateau cells (as opposed to non-nutrient deficient cells), found them fully capable of repairing sublethal as well as potentially lethal injury (4, 10).

After reviewing the literature, it has become apparent that the nutritional state of the plateau-phase cells is quite important. This is best realized when we examine the results of Hahn (4). He reports that non-nutrient deficient (fed), plateau phase Chinese Hamster cells completely lack the ability to repair either sublethal or potentially lethal damage. However, as mentioned above, he finds nutrient deficient (unfed) plateau phase cells capable of such repair. In our laboratory, the plateau phase cells we have used would be classed as non-nutrient deficient. This importance is also emphasized by Mauro (4). He found survival curves of HeLa cells irradiated in the plateau phase lack a shoulder if a combination of horse and human serum is used to supplement their growth medium. But, if fetal bovine serum is used, a small shoulder is present. Mauro also reports a difference in the  $D_0$

of plateau cells that is dependent upon the type of serum used in the culture medium.

The magnitude of the extrapolation number is a measure of the amount of sublethal damage a cell can sustain. In many experimental situations it has also been an indication of repair. The classical technique for measure of repair is the split dose experiment (39). We used this technique to examine the repair of sublethal damage in RT-2 cells in exponential and plateau-phase of growth.

In this procedure, plateau-phase cells are trypsinized, diluted, and plated in 60 mm petri dishes. A portion of them are exposed to 500 rads irradiation after allowing 24 hours to attach and regain exponential growth. At various times after the initial exposure, a challenging dose of 500 rads is given. The remaining portion of the plateau-phase cells are given 500 rads immediately after plating, and challenged at the appropriate time afterwards with another 500 rads. The plates are incubated for 9 - 14 days and the plates are fixed and the colonies stained and scored. As shown in Figure 13, survival is plotted on a semi-log scale as a function of separation time of the initial and the challenging doses of radiation. Log-phase cells demonstrate the ability to repair sublethal injury with a repair half time ( $T_{1/2}$ ) of about 90 minutes. Plateau-phase cells also exhibit this ability, but with a longer  $T_{1/2}$  (Figure 14). The delay in the time required to reach maximum survival as shown by the plateau phase cells very likely reflects the time required for plateau cells to regain exponential characteristics.

Using non-nutrient deficient cultures for split dose experiments, Hahn reports a total lack of repair of sublethal injury (4). Similar

principles were also stated by Revesz and Littbrand working with a different line of Chinese Hamster cells (40). Little, however, using dense cultures of Chang liver cells found no reduction in repair capacity (41). Chapman, et al., studying Chinese Hamster cells (line V79-379-A) resuming growth after plateau, report an increase in radiation resistance (above that in plateau) related to an increase in extrapolation number (6). They found these "growth resumption" cells able to recover from sublethal injury, and did so within 2 hours after being removed from plateau-phase.

Since the results of our split dose experiments may have been influenced by our method of handling plateau-phase cells, we have started new experiments designed much as those of Hahn (4). This involves irradiating the plateau-phase cells in the culture vessel prior to disturbance of the monolayer. After treatment, the cells are immediately plated and at the appropriate time, the challenging dose applied. Preliminary results indicate that the plateau cells have a marked reduction in their ability to repair sublethal injury. However, they still exhibit a modest increase in survival after a 4-hour separation of 500 + 500 rads. Though not conclusive yet, these results seem to indicate at least a qualitative similarity to Hahn's Chinese Hamster cell system.

Another important consideration that must be made of plateau-phase cells is the question of repopulation. Are cells which have been maintained in plateau-phase for an extended period of time able to repopulate a system? This is best answered by the fact that the

plating efficiency of plateau-phase RT-2 cells is nearly the same as that of cells used in exponential growth. In all of our experiments, we realized plating efficiencies of from 50 - 90% for exponentially growing cells. The plating efficiencies observed for plateau-phase cells are from 40 - 85%, with the majority in the 60 - 80% range. This then would indicate a high degree of viability as cells move from plateau to exponential growth. Furthermore, examination of growth curves derived from cells previously held in a plateau-phase for several days indicates a doubling time of the same duration as that obtained from cells used in exponential growth. The plateau cells do seem to have a slightly longer lag period before attaining exponential growth. This is the extent of the work we have finished using plateau-phase RT-2 cells. We have in progress experiments designed to measure the plateau-phase cell's ability to modify potentially lethal radiation injury, and further experiments on the repair of sublethal injury. We are also continuing to characterize the response of the exponentially growing RT-2 cell to radiation damage under a variety of conditions. Tables V and VI summarize the biologic and radiobiologic characteristics of the RT-2 cell line and compare these data with those of other workers using different cell lines.

We have measured the rejoining of radiation induced DNA breaks in exponentially growing RT-2 cells as well as in the *in vivo* rat sarcoma (42). The results are very similar to those obtained from CHO cells (Figures 2 and 4) and L-cells (13, 14). The largest number of DNA breaks appeared immediately after irradiation. With time (10 minutes),

BIOLOGIC PROPERTIES

Cell Line	T <sub>C</sub>		Cell Cycle		T <sub>M</sub>		Growth Fraction in Plateau		Nutritional State in Plateau	Viability		Ref
	Log	Plateau	Log	Plateau	Log	Plateau	Log	Plateau		Early Plateau	Late Plateau	
CHO-Hahn	15	32	6	18	1.00	Unfed	>75%	>78%	>50%	44		
CHO-Hahn	15		6	6	1.00	Fed	>75%	Slow decline to 30-40%		4, 3 44		
CHO-Dalrymple	15	--	--	--	1.00	Fed	>65%					
RT-2 Dalrymple	21	>72	8	>25	1.00	Fed	>70%	>60%	--	8		
LICH-Little	24	--	--	--	1.00	Fed	>60%	>60%	--	10, 41		

RADIOBIOLOGIC PROPERTIES

Cell Line	Extrapolation Number <sup>n</sup>		D <sub>0</sub>		Repair of Sub-lethal Radiation Injury		Rejoining of DNA Breaks		Ref.
	Log	Plateau	Log	Plateau	Log	Plateau	Log	Plateau	
CHO-Hahn (fed)	2.8	1.0-2.0	142	135-148	Yes	Diminished	--	--	4,5
CHO-Hahn (unfed)	2.8	1.8-3.9	142	127-148	Yes	Yes	--	--	4
CHO-Dalrymple (fed)	2.7	1.0	140	140	Yes	Diminished	Yes	Yes	8,14
RT-2-Dalrymple	2.7	1.6	230	250	Yes	Diminished	Yes	Yes	8,42
LICH-Little	2.1	3.8	152	123	Yes	Apparently the same, or higher	--	--	10,41

the number of these breaks decreased to control level as a consequence of rejoining. Again, the presence of DNP prevented the appearance of DNA breaks above control levels.

The RT-2 cell line can be synchronized with a high degree of synchrony by use of the detachment method described by Tobey and Anderson (43). We plan to measure the radiation response of RT-2 cells in various portions of the cell cycle. Work is also in progress to determine the effect of hypoxia on both plateau and log phase cells. From the data we have already collected, and from the information from experiments in progress, we feel the development of the RT-2 cell line has great potential. A culture system which can be handled with great ease, either *in vivo* or *in vitro*, in exponential and plateau growth, and also has a high malignant potential should be of great value in the study of the response of neoplastic tissue. The fact that it can be utilized as a cell renewal system that is relatively stable can only add to its value.

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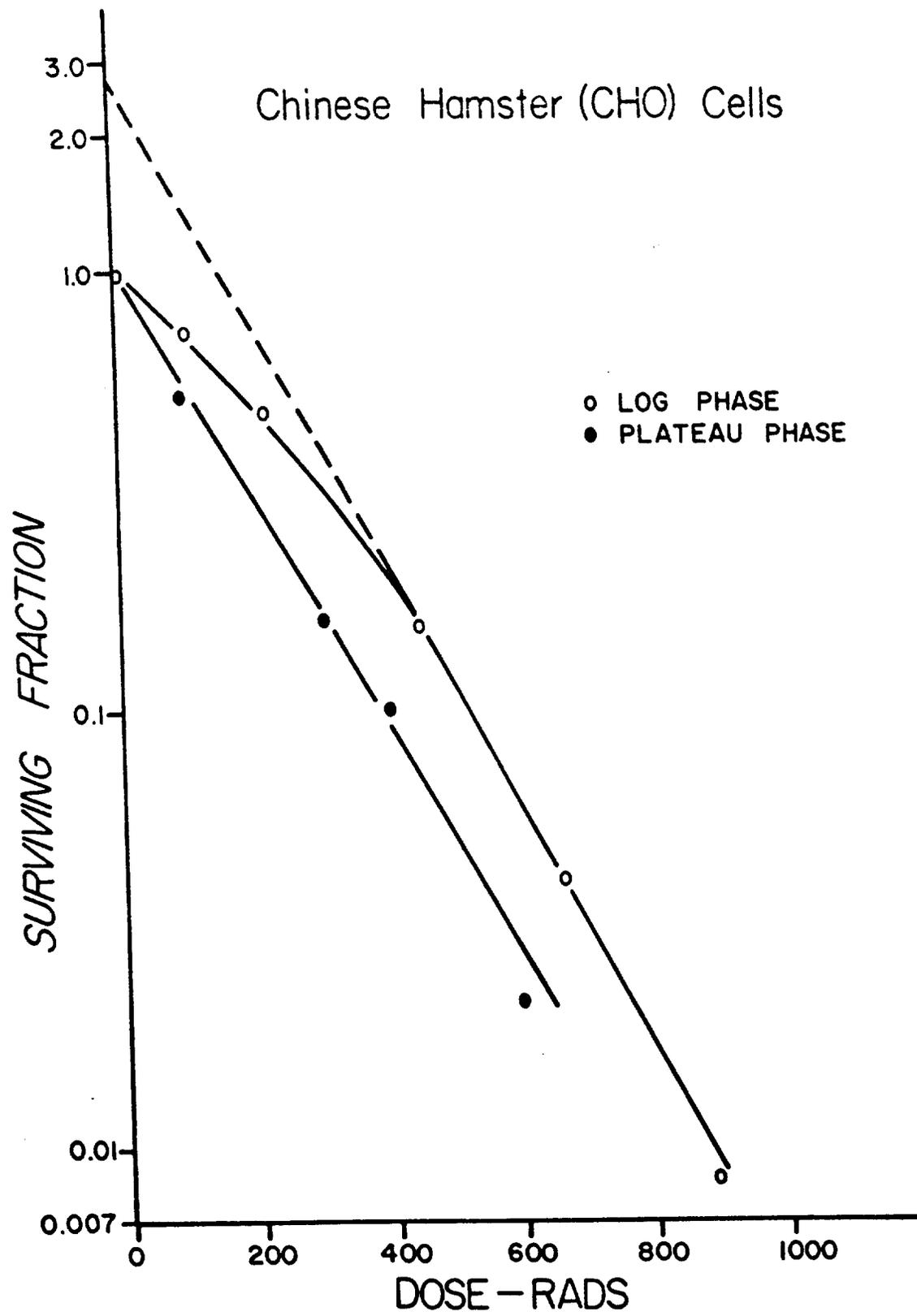
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CAPTION TO FIGURE 1

Comparison of the single dose response of plateau-phase and exponentially growing CHO cells. Notice the marked depression of the extrapolation number for the plateau-phase cells. The slopes, however, are nearly parallel.

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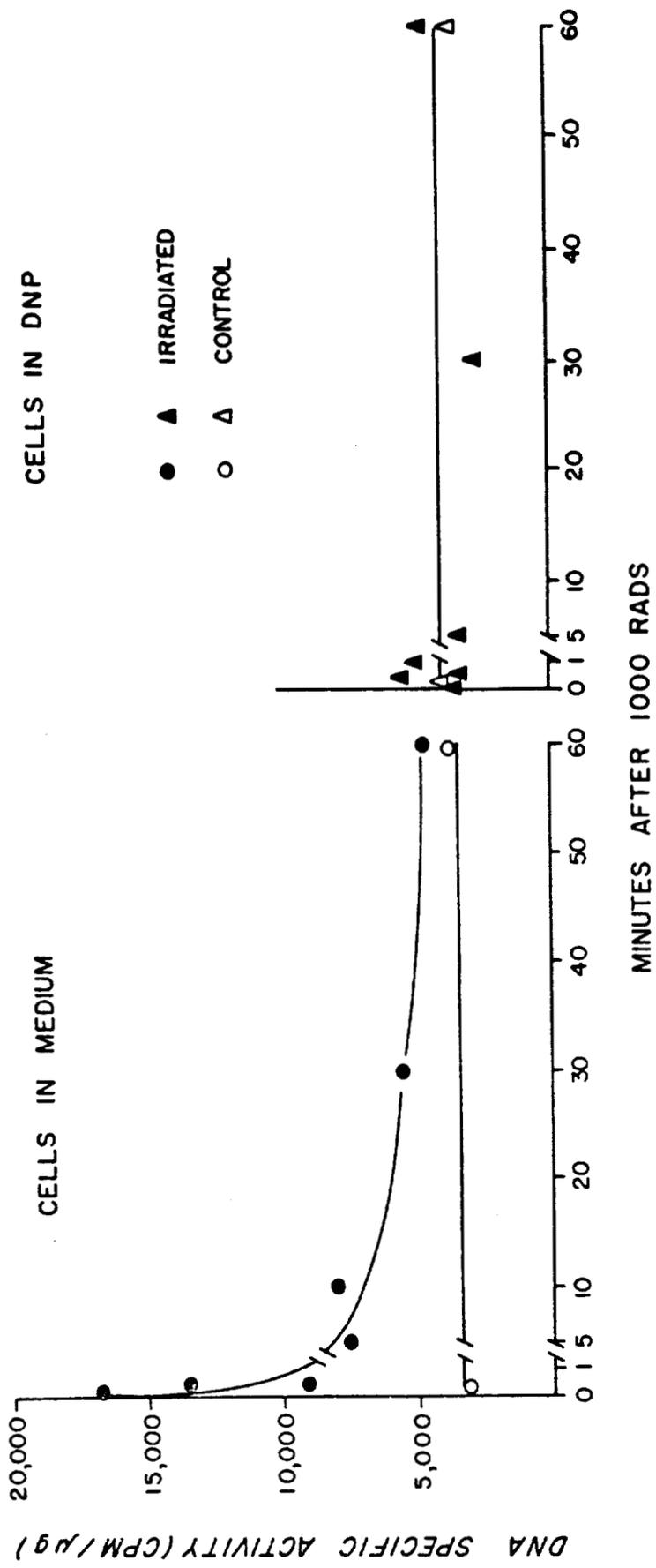


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CAPTION TO FIGURE 2

DNA rejoining by CHO cells in exponential growth. The cells shown in the left panel were suspended in medium. The cells shown in the right panel were suspended in  $10^{-4}$ M DNP. Rejoining was measured by the polynucleotide kinase method. The dose was 1000 rads.

# CHINESE HAMSTER (CHO) CELLS

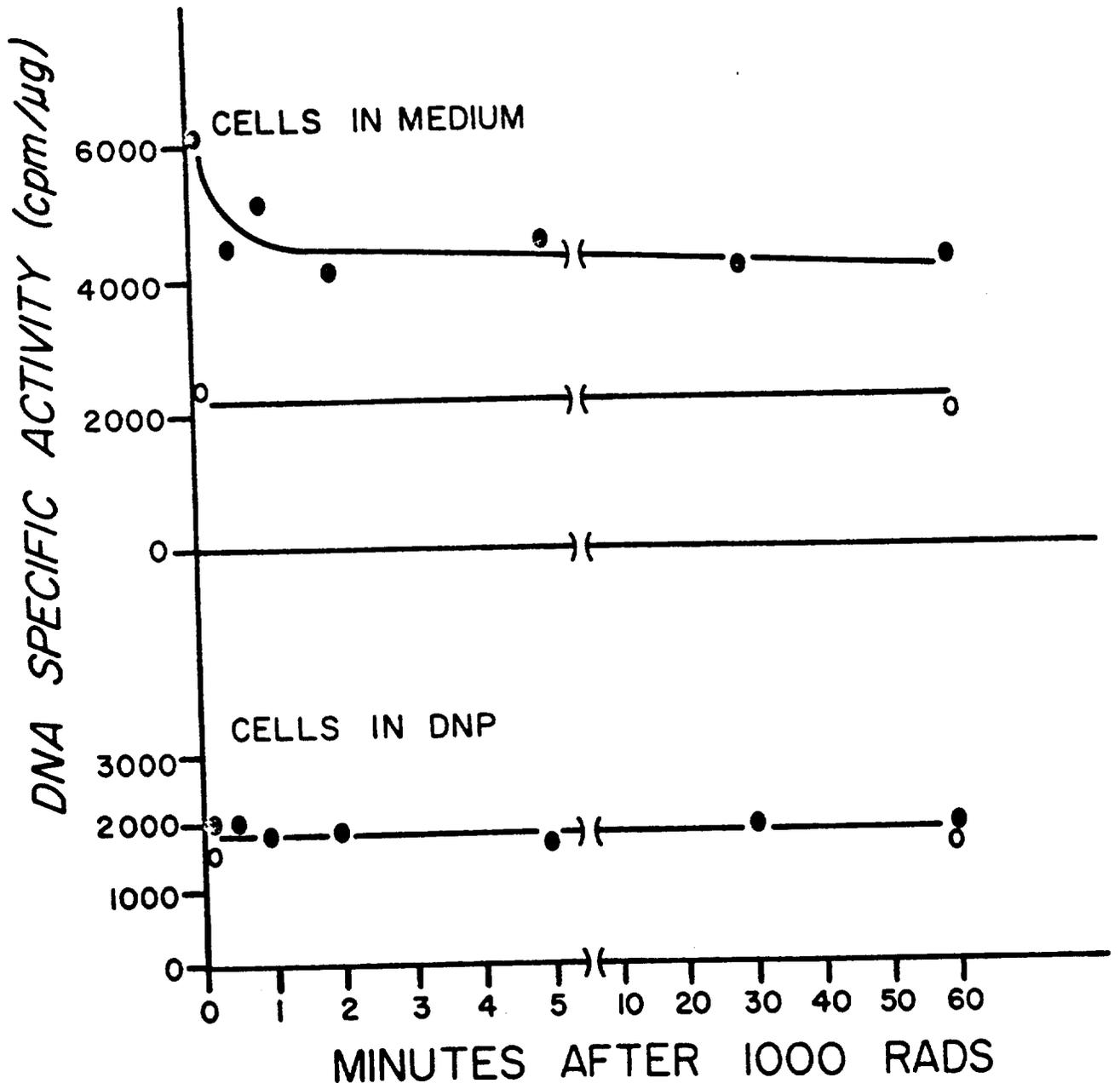


CAPTION TO FIGURE 3

The upper portion of the figure shows the kinetics of rejoining of  $5'\text{Po}_4$  termini by plateau-phase cells which were suspended in medium. Notice that the number of these termini (closed circles) was everywhere greater than for central plateau-phase cells (open circles). The lower panel, however, shows that suspending the cells in  $10^{-4}\text{M}$  DNP prevented the appearance of  $5'\text{Po}_4$  termini after irradiation (open circles) as compared with control (closed circles).

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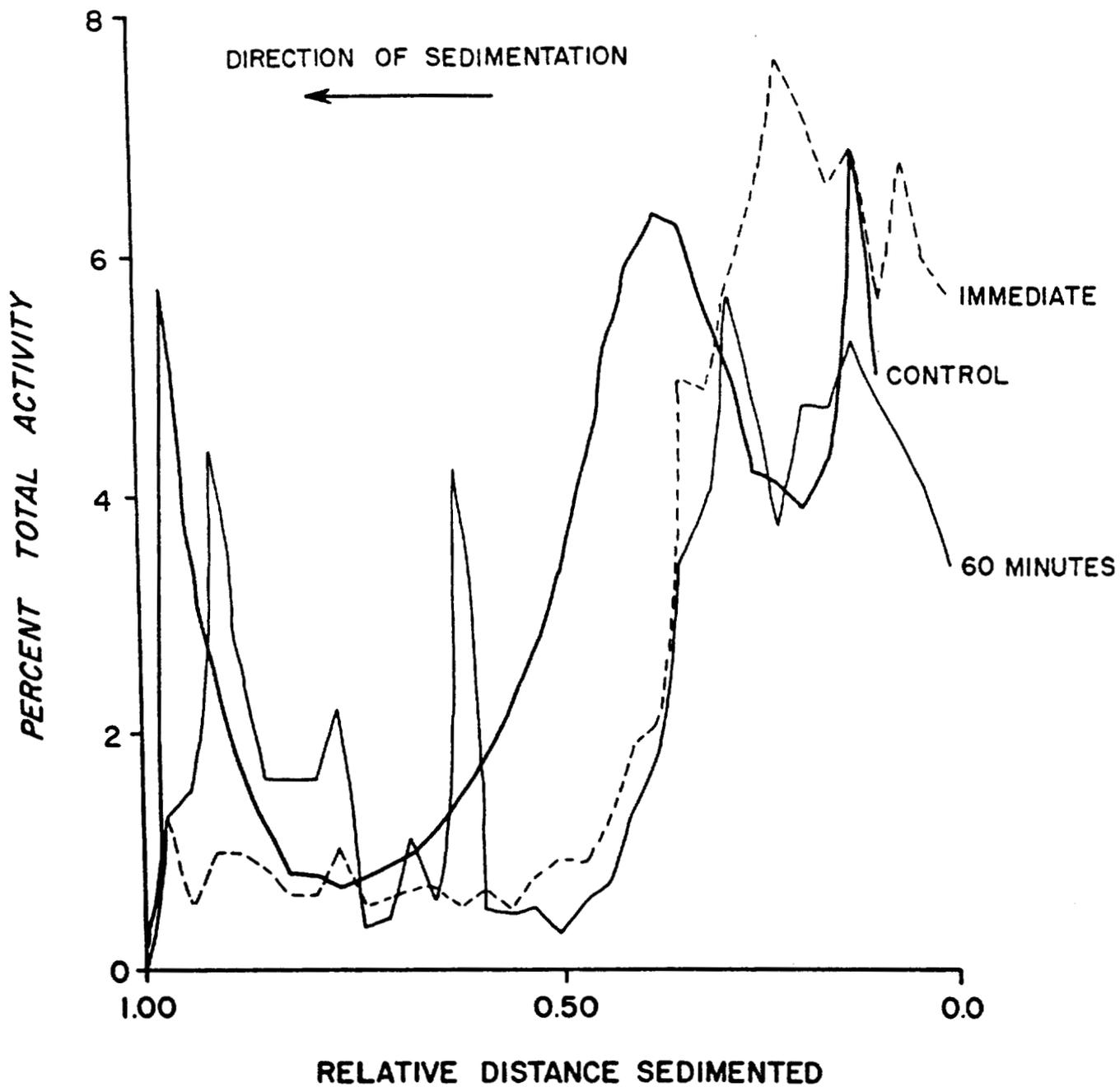
# PLATEAU PHASE CHO CELLS



CAPTION TO FIGURE 4

DNA rejoining by plateau-phase CHO cells as measured by alkaline sucrose sedimentation. As the figure shows, the control was heavier than the irradiated (10,000 rads) DNA (see text). Also, no evidence of rejoining was detected for the cells lysed one hour after irradiation, as compared with those lysed immediately after exposure.

CHINESE HAMSTER (CHO) CELLS  
PLATEAU PHASE



CAPTION TO FIGURE 5

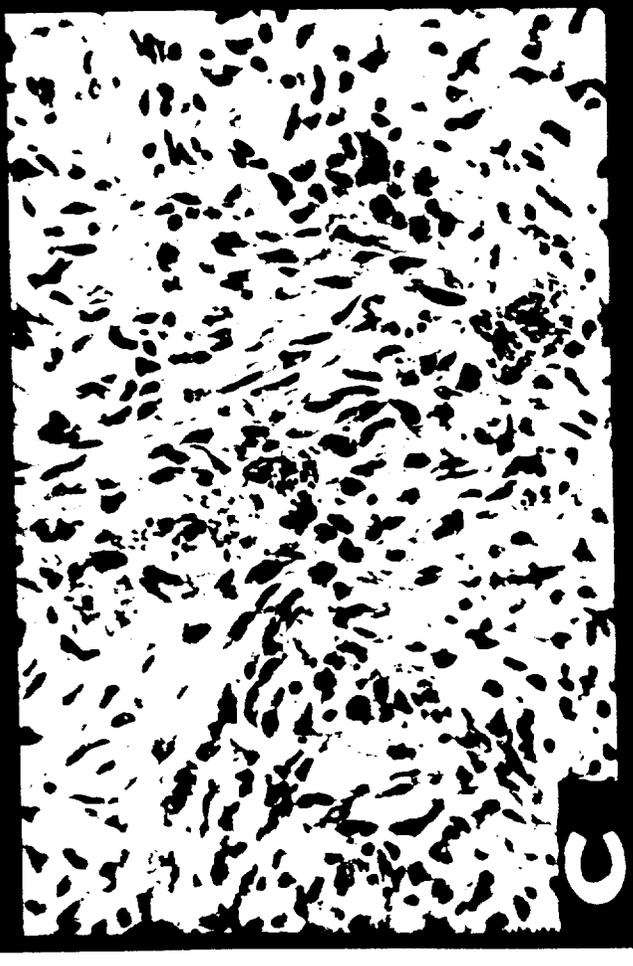
The Rat Sarcoma (RT-2) system. In Plate A is a young Fischer rat exhibiting a well developed tumor. The gross tumor is shown in Plate B. A histologic section of such a tumor is shown in Plate C. The RT-cell as grown in culture (monolayer) is shown in Plate D.



**A**

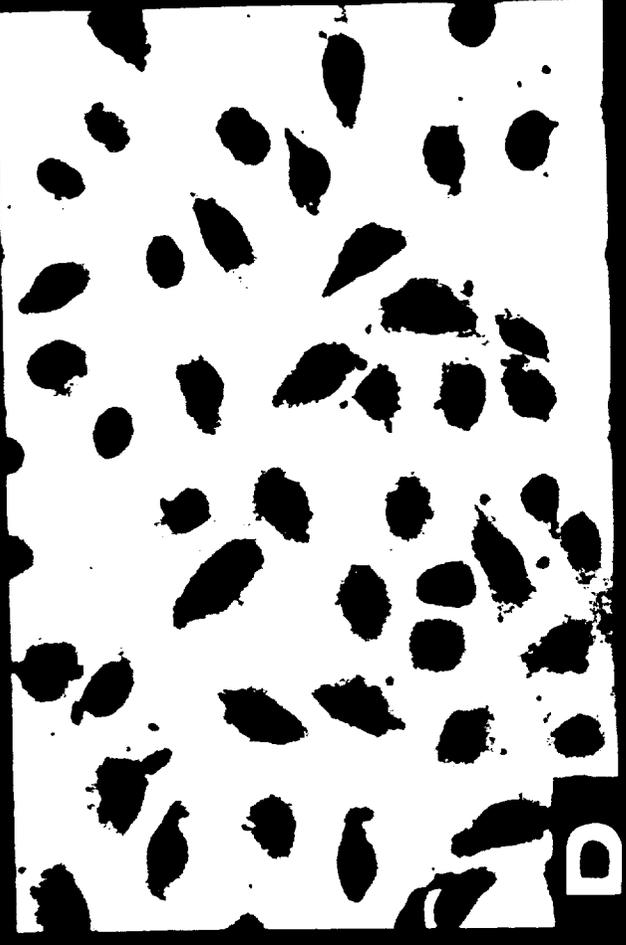


**B**



**C**

IN VIVO (X400)



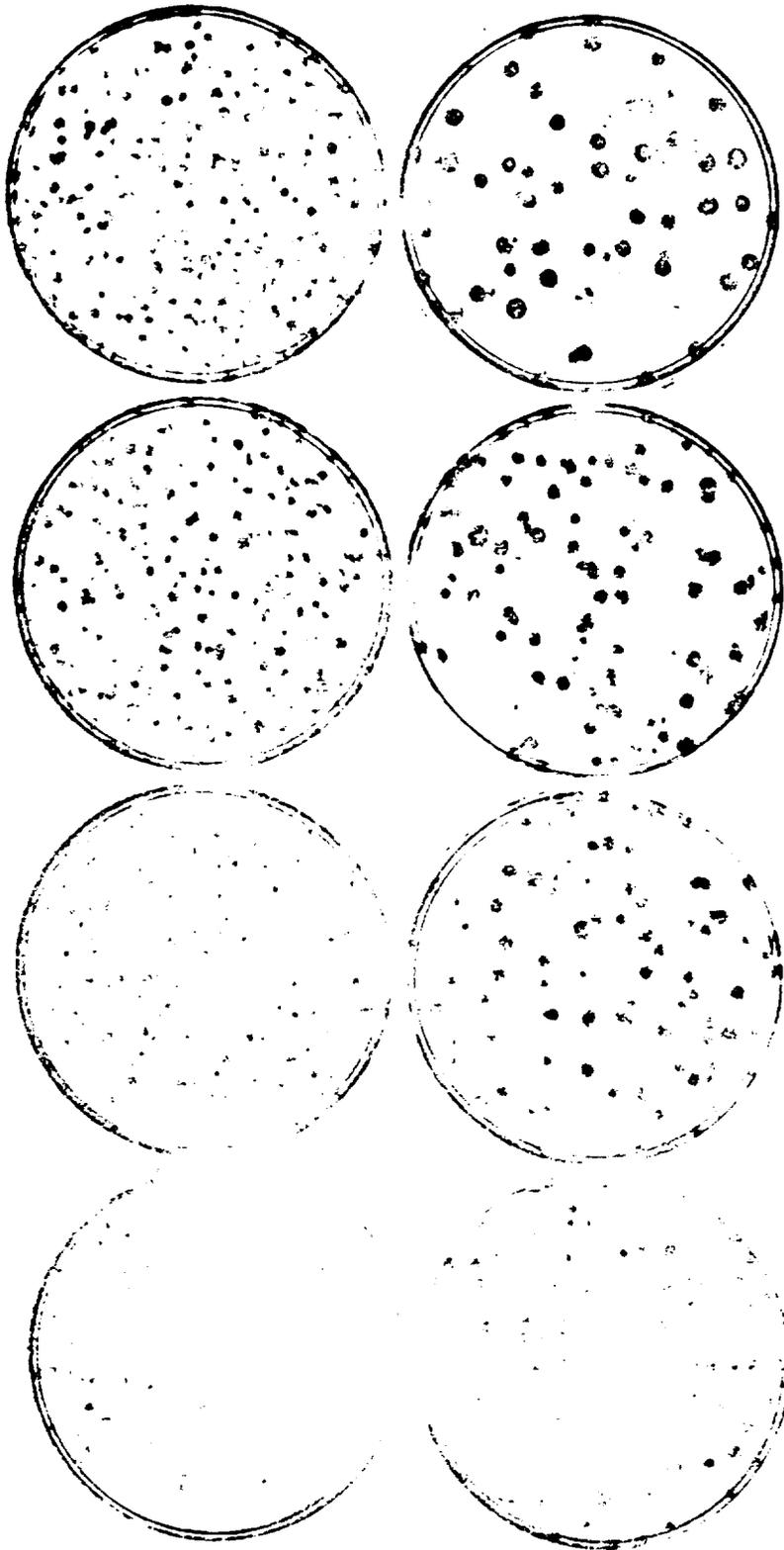
**D**

IN VITRO (X400)

CAPTION TO FIGURE 6

Colonies derived from single RT-2 cells after graded doses of X-rays.  
Note the well formed, compact colonies even after X-ray doses from  
zero (lower right) to 1000 rads (upper left).

1138370

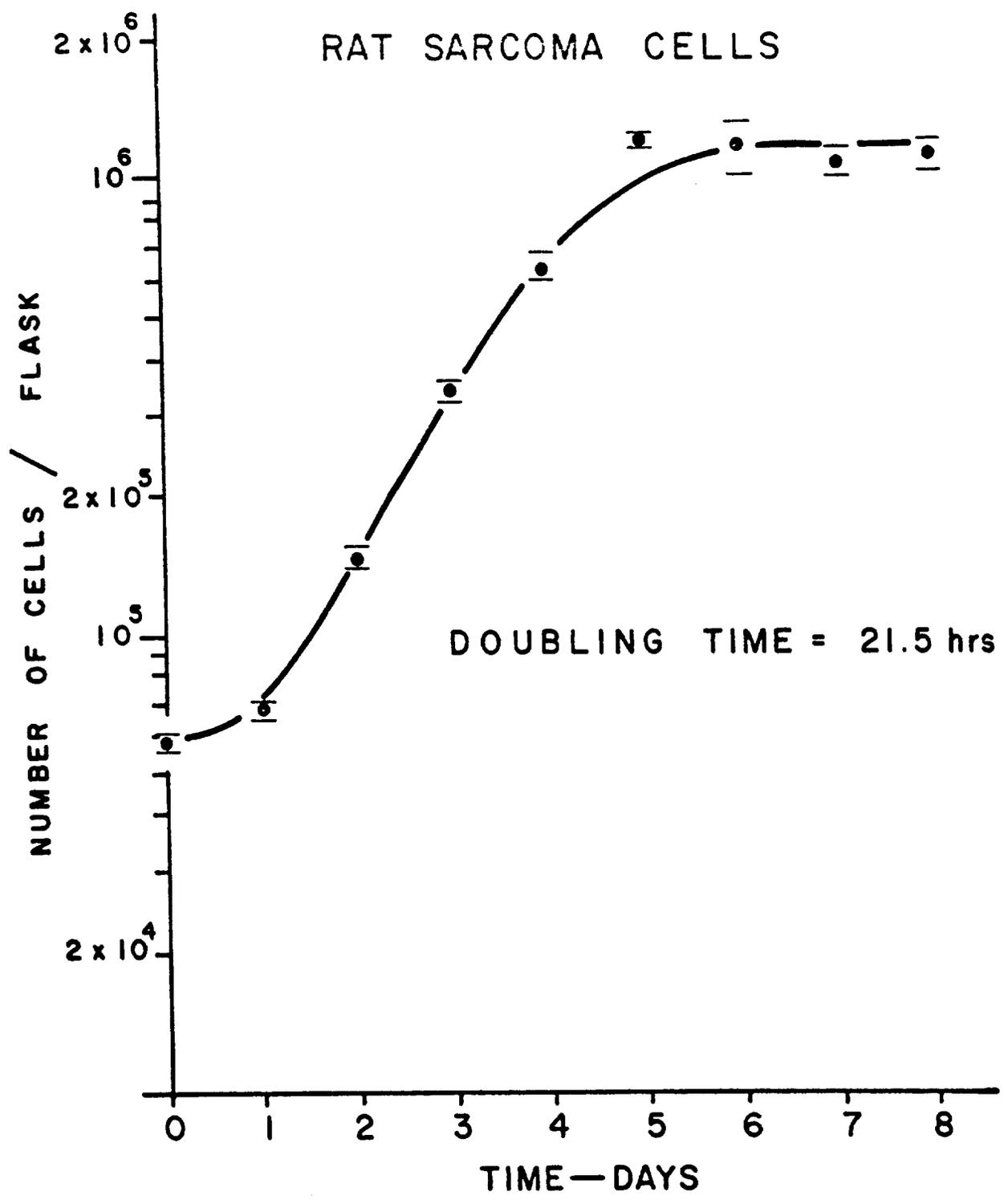


1138371

CAPTION TO FIGURE 7

A typical growth curve of the Rat Sarcoma (RT-2) cell line. The cells while in exponential growth have a doubling time of about 21 hours. A plateau-phase is established after about 5 days of culture. Though not shown in this figure, the plateau-phase may be maintained for several more days by changing medium daily after day 5.

1138372



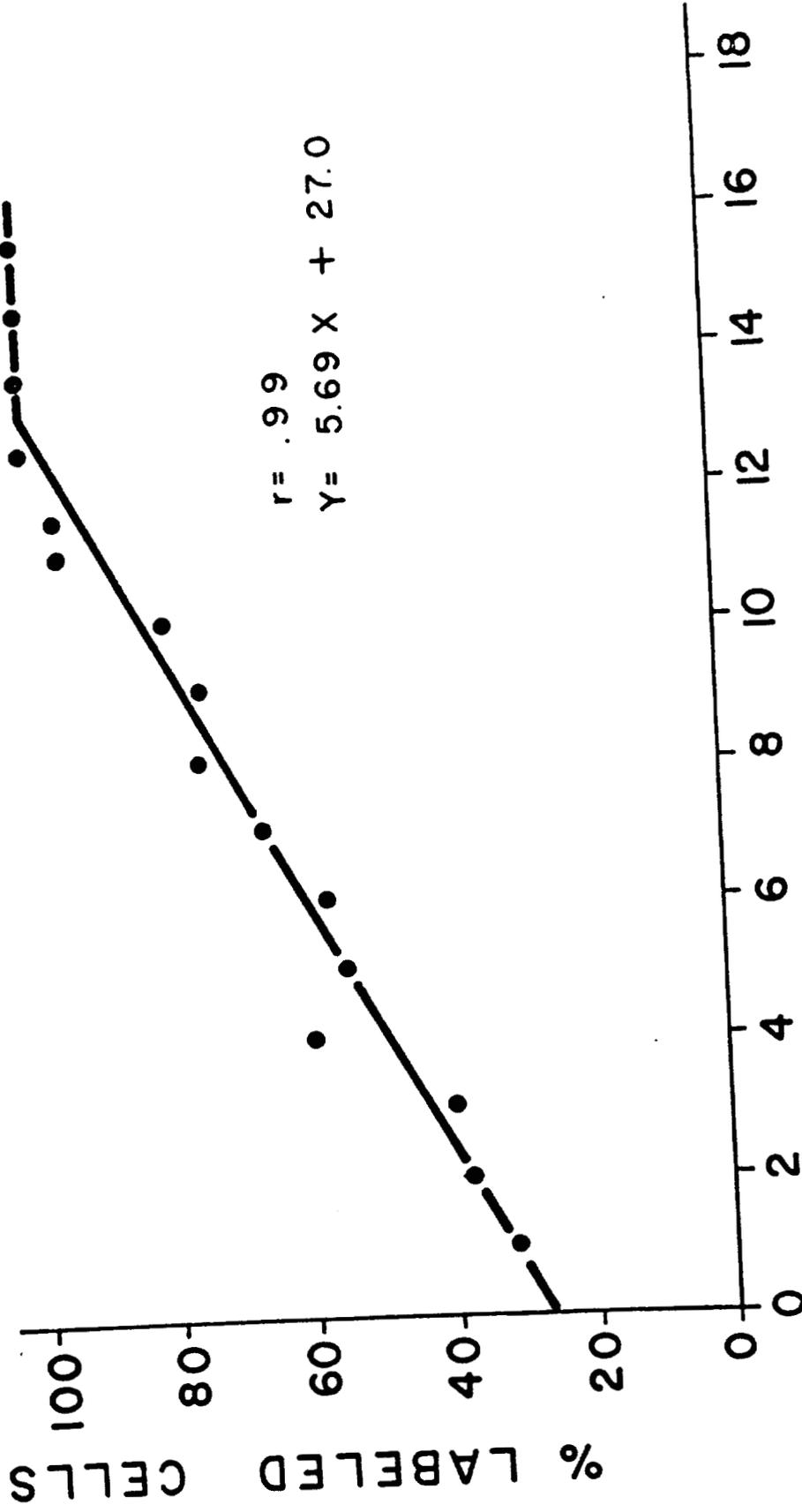
1138373

CAPTION TO FIGURE 8

The percent labeled cells is plotted as a function of the time of a continuous exposure to  $^3\text{H}$ -thymidine. The curve intersects the ordinate at the initial labeling index (size of the S pool, see text) and reaches 100 percent after  $T_{G1} + T_M + T_{G2}$ . Cultures were given 1 uci/ml of medium (specific activity of 20.1 ci/mM) and exposed to Kodak NTB-3, nuclear track emulsion for 41 days.

1138374

# RT-2 CELLS

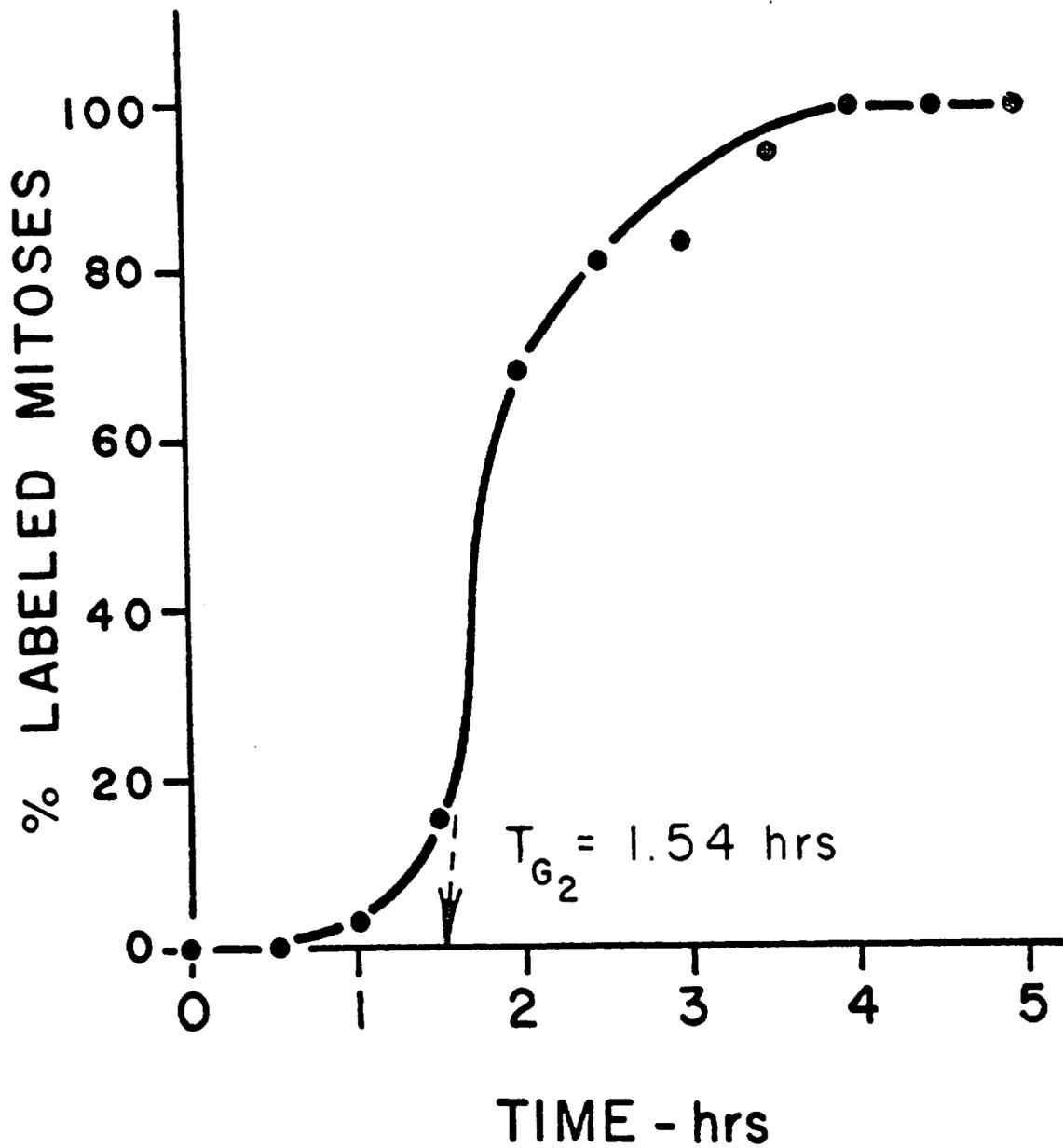


EXPOSURE TIME - HOURS

CAPTION TO FIGURE 9

The percent labeled mitoses (labeled mitoses/total mitoses) is plotted as a function of time after a pulse label of  $^3\text{H}$ -thymidine. Extrapolation of the first ascending limb to the abscissa gives a  $T_{G2}$  value of 1.54 hours (see text).

# RAT SARCOMA CELLS

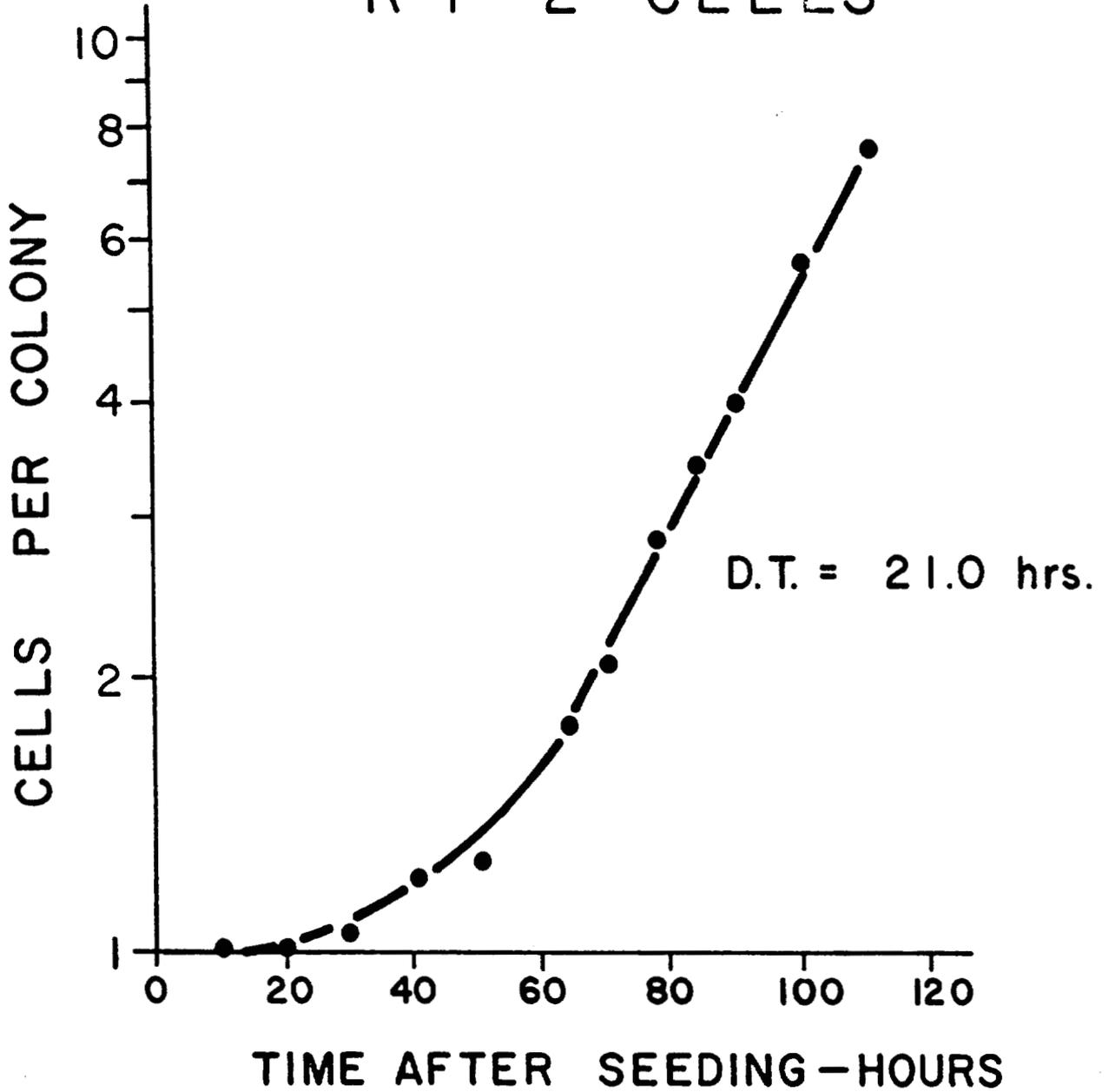


CAPTION TO FIGURE 10

A typical growth curve for the RT-2 cell line. This growth curve was generated by clone size analysis. The number of cells/colony is plotted as a function of time after seeding. The doubling time as determined from the log-linear portion of the curve is 21 hours.

1138378

# RT-2 CELLS

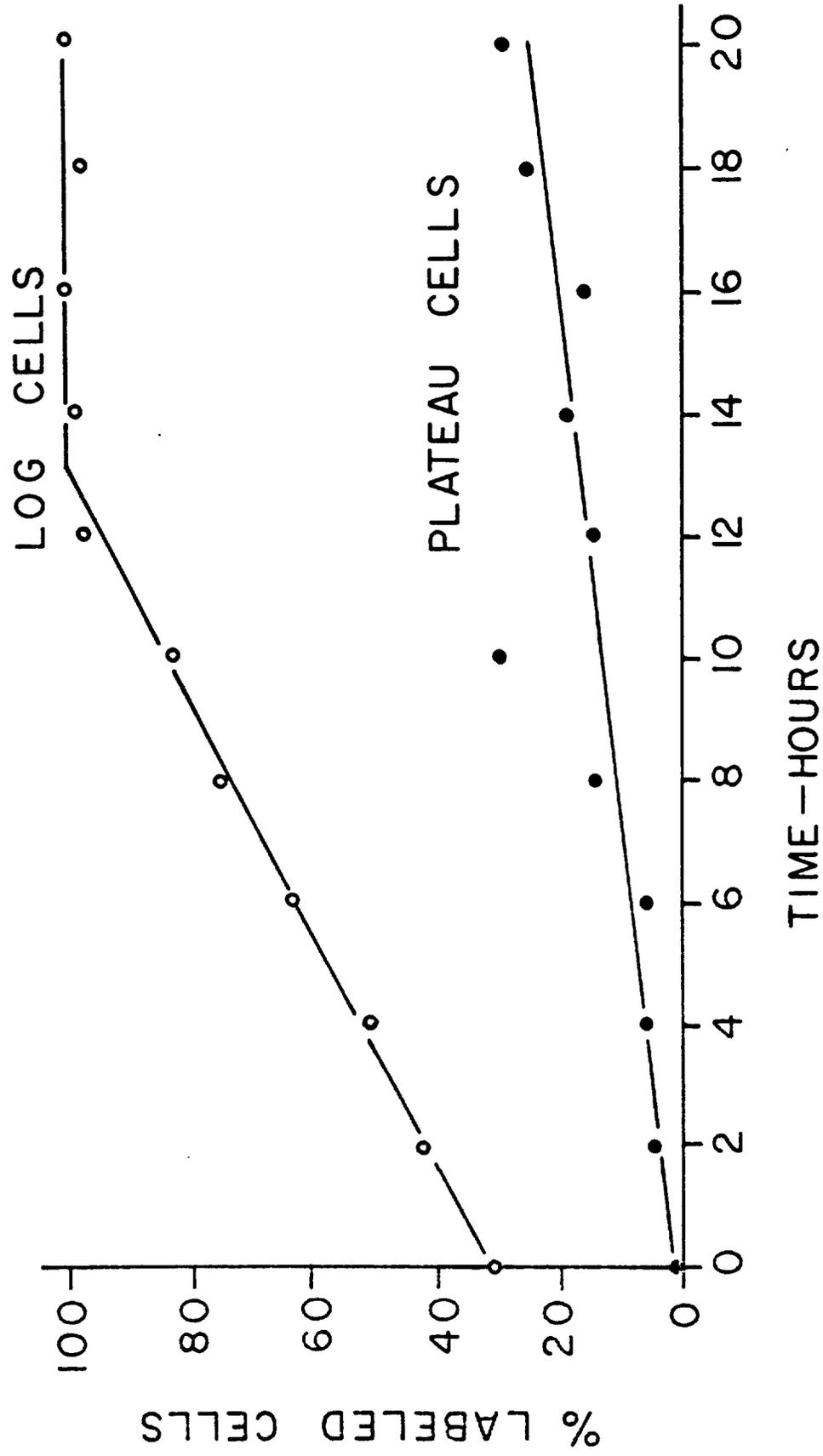


CAPTION TO FIGURE 11

Comparison of the labeling profiles of plateau and log phase RT-2 cells. The upper curve shows the percent labeled log-phase cells (actually labeled nuclei) as a function of time after addition of  $^3\text{H}$ -thymidine. The bottom curve shows the percent labeled plateau-phase cells as a function of time after addition of  $^3\text{H}$ -thymidine. The upper curves reached 100 percent labeling after about 13 hours. Though not shown, the bottom curve approached 91 percent labeling after 72 hours. The exposure to label was continuous in both cases.

1138380

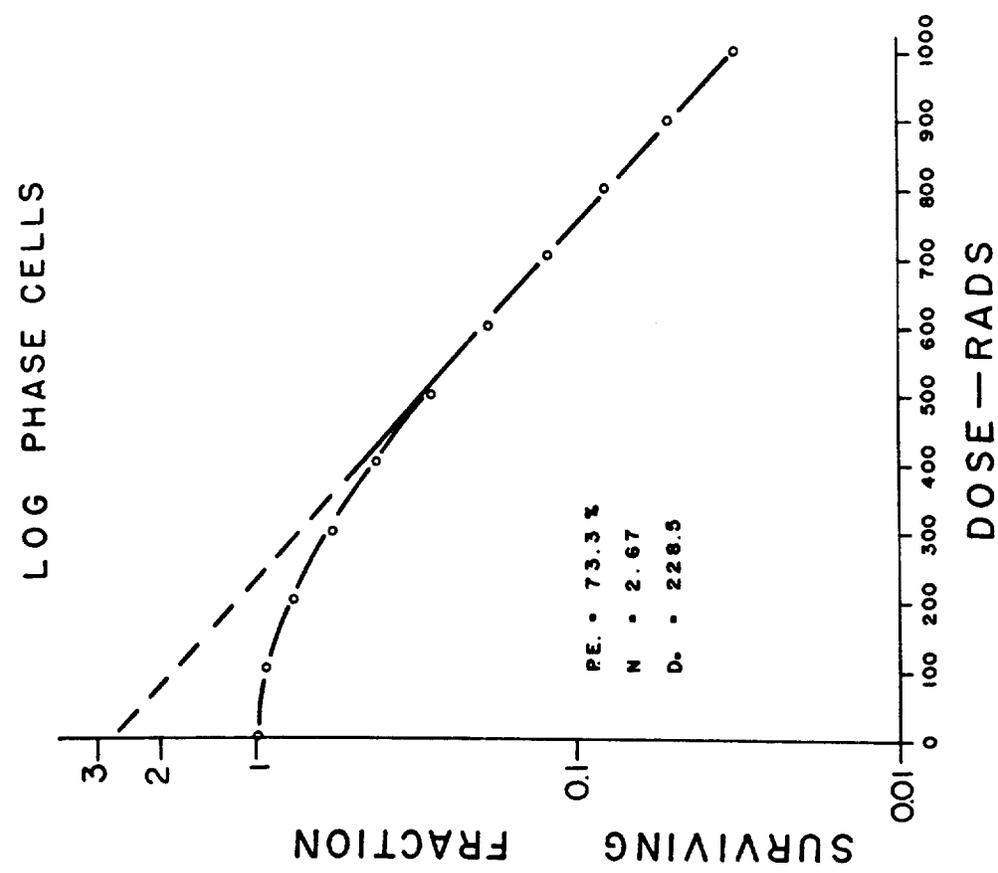
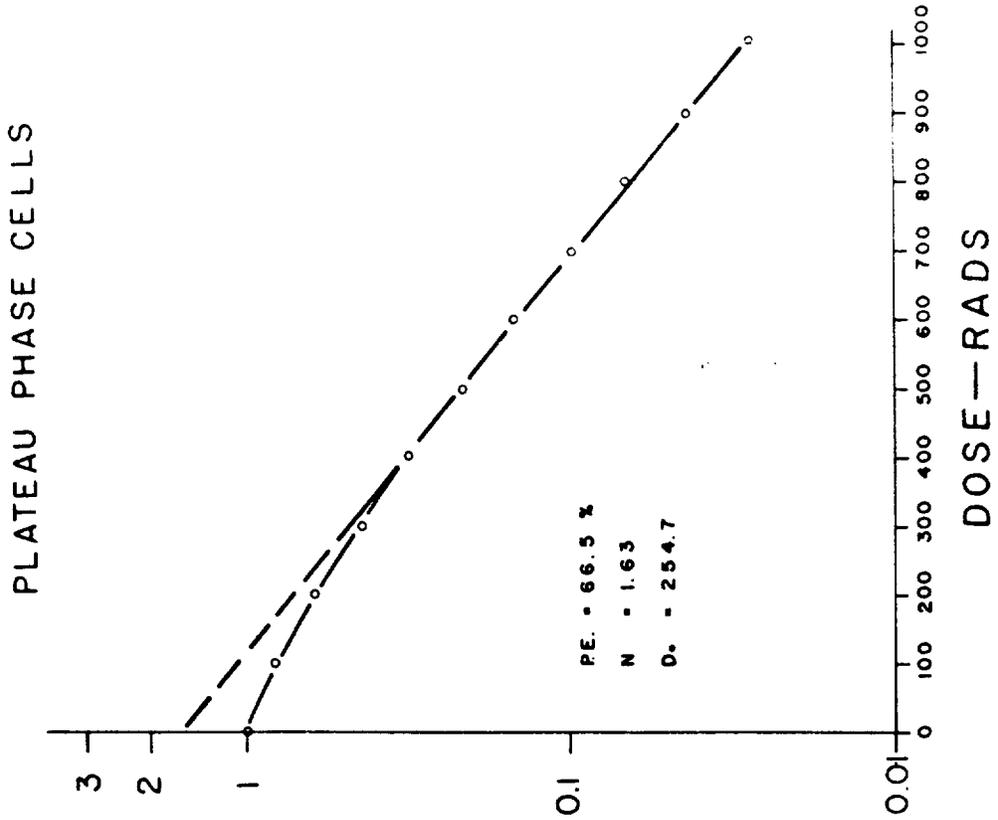
# RAT SARCOMA CELLS



CAPTION TO FIGURE 12

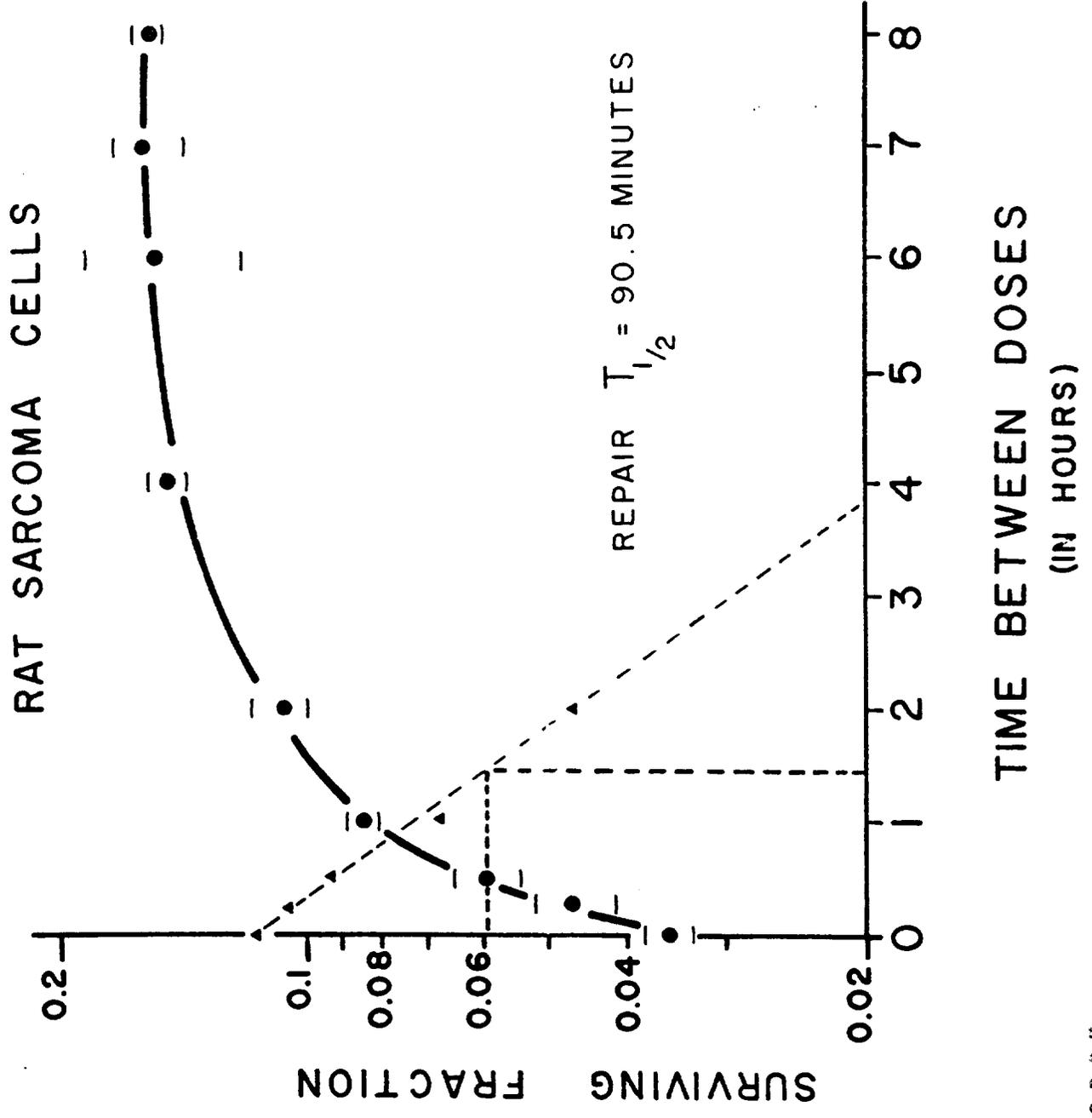
Comparison of the single dose response of plateau-phase and exponentially growing RT-2 cells. Notice the reduction in extrapolation number for the plateau-phase cells. The slopes are approximately the same.

# RAT SARCOMA



CAPTION TO FIGURE 13

A measure of the magnitude of repair of sublethal injury by RT-2 cells in exponential growth. This curve shows the paired dose response of log-phase cells. Doses given were 500 + 500 rads. The magnitude of the survival increase was a factor of 5 with a repair halftime ( $t_{1/2}$ ) of about 90 minutes.



CAPTION TO FIGURE 14

A measure of the magnitude of repair of sublethal injury by RT-2 cells in plateau-growth. This curve shows the paired dose response of plateau-phase cells. Doses given were 500 + 500 rads. The survival increased by a factor of 2-1/2 with a repair halftime ( $t_{1/2}$ ) of about 105 minutes.

# RAT SARCOMA CELLS—PLATEAU PHASE

