AN INVESTIGATION OF
RADIATION-INDUCED MITOTIC INHIBITION
IN L-STRAIN MOUSE CELLS

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RADIATION-INDUCED MITOTIC INHIBITION
IN L-STRAIN MOUSE CELLS

by

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SCOPE AND CONTENTS: The variation in sensitivity of L60T cells to gamma rays has been studied as a function of position in the cell division cycle. For a dose range of 0-12,000 rads, no significant variation was found for mitotic delay. Such was not the case for sensitivity to cell killing, which was found to increase as the cells passed from G_1 through S to G_2 of the division cycle. The results of mitotic delay are in disagreement with results published by other workers although the survival data agree with previous reports for a similar cell line. Results reported in connection with cell cycle determinations and mitotic delay suggest that the existence of a repair cycle operating concurrently with the normal cell cycle may be postulated. The theoretical treatment of mitotic delay given by Lea is examined and is not found to describe adequately the present results. Finally, the evidence reported here suggests that mitotic delay and radiation lethality are not separate manifestations of the same phenomenon. Experimental materials for further investigation into the repair processes involved are suggested.

PREFACE

This thesis describes studies carried out in the Research Unit in Biochemistry, Biophysics and Molecular Biology at McMaster University, from June 1964 to June 1967.

Sincere thanks are due to Dr. D. O. Schneider for his valuable advice and encouragement in the supervision of this project. The author is grateful also to the National Cancer Institute for its generous financial support.

Research Unit in Biochemistry,
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I. INTRODUCTION

For a number of years there have been intensive investigations into the effects of ionizing radiations on living cells. The qualitative and quantitative responses of cells to radiation have been well documented, especially with respect to cell death and genetic and cytological effects.

The implications of investigating the effects of ionizing radiations on mammalian cells are apparent in the developing techniques of radiation therapy for cancer. In a broader sense, the radiation response of cells is useful as a tool in exploring the molecular basis of some cell processes. For example, the problem of the cell's control of mitotic activity remains unsolved and, since mitotic inhibition is the temporary loss of the cell's ability to undergo mitosis following irradiation, a study of this phenomenon may focus directly on the control mechanism.

Following the absorption of ionizing radiation by cells, several distinct cytological effects are observed. The first of these is mitotic inhibition, the immediate, temporary decrease in the fraction of cells undergoing mitosis in a population. Subsequently chromosomal aberrations and cell death are observed.

As a quantitative measure of mitotic inhibition, the time between the irradiation and the reappearance of mitotic activity is defined as the mitotic delay. This is effectively a measure of the duration of the mitotic inhibition. Data from a number of organisms indicate

that, when mitotic delay is plotted against the logarithm of the dose, a straight line is obtained. Lea has sought to explain this relationship on the basis of the concept of cumulative dose, and has developed a mathematical treatment for this model (1).

It has been claimed by Puck (2) that mitotic delay, chromosomal aberrations, and cell death are caused by the same initial lesion which he designates as a primary chromosomal injury. The work of Whitfield et al. (3, 4, 5) would tend to favour a chromosomal effect for mitotic delay. They found that the addition of chromatin condensing agents (agmatine, Ca^{++} salts, hypertonic salt solutions) to an irradiated culture caused a decrease in the mitotic delay. It was suggested that the elongation of the chromosomes of cells about to enter mitosis is the cause of the delay following irradiation, and that the condensing effect of these agents on the chromatin material may compensate for this radiation response.

In addition, several workers have reported a correlation between cell killing and chromosomal aberrations (6, 7, 8), the cause of death at division being attributed to the formation of chromosome bridges or genetic unbalance following division. Thus there would appear to be a common involvement with the chromosome in these effects. However, investigations into the relative biological efficiencies (R.B.E.) of radiations of various linear energy transfers (L.E.T.) have shown that the maximum biological effect for mitotic inhibition occurs at an L.E.T. significantly different from that for cell death (9, 10). The immediate inference from this is the existence of two different sites of action for the radiation. It is possible that both of these sites

are associated with the chromosome, but that cell death and mitotic delay are caused by the same lesion is doubtful.

The existence of cell processes responsible for repair of radiation damage has been shown experimentally in recent years. Elkind and Sutton (11) irradiated Chinese hamster cells with two doses separated by various incubation times, and demonstrated recovery between the doses. Since several workers (6, 12, 13) have found that the sensitivity of cells to radiation killing is a function of their age in the cell cycle, this recovery might be due solely to a progress of the cells to a different sensitivity state during the incubation between doses. Whitmore et al. (13) have managed to separate the effects of true repair and cell progression, however, and have attributed the recovery curves to a combination of both effects.

The existence of repair functioning in the release of cells from mitotic inhibition is self-evident from the very description of the phenomenon. Lea's model of cumulative dose incorporates a repair system which causes an exponential decay of the radiation damage and ultimately permits the cells to resume mitotic activity.

As has been mentioned, several workers have examined the differential sensitivity of cells irradiated at different times of the division cycle, in attempts to describe more fully the repair between fractionated doses. In a great many cell systems, the life history of a cell can be divided into four distinct stages (14). Following mitosis there exists a period during which DNA synthesis does not occur (G_1), a subsequent period of DNA synthesis (S), a second period during which DNA is not synthesized (G_2) and, finally,

a period of mitosis (M). The mitotic phase can be observed cytologically while the S period may be determined by the fraction of cells which incorporate a pulse of radioactive thymidine.

The present investigation is concerned with the variation of the mitotic delay response with cell age, in order to make a comparison with the results obtained for cell killing. Since a repair system is operative in both phenomena, it is of importance to determine dose-response curves for both, to discover whether or not common features exist. A demonstration that mitotic inhibition follows a sensitivity parallel to that for cell death throughout the cycle, would be evidence in support of Puck's hypothesis involving a primary chromosomal lesion. If at any stage in the cycle the sensitivities were shown to diverge, the same hypothesis would be thrown into doubt.

Variations in the mitotic delays of cells irradiated at different times in the cell cycle have been reported (6, 12) but only very recently have Whitmore et al. published dose-response curves for this effect (15). Their results for radiation doses up to 1100 rads indicate that sensitivity to mitotic delay increases as the cells move from G_1 to S to G_2 through the cell division cycle. The present investigation extends the range of doses used to 12,000 rads. Over this range no significant variation in sensitivity to mitotic inhibition around the cell cycle is found, in contradiction to the conclusions drawn by the above workers. Moreover, the theoretical treatment for mitotic delay given by Lea (1) is evaluated and is shown to be inapplicable to the present results, except possibly over a small dose range.

An interdependence of the generation time, the length of the G_1 phase and the mitotic delay has been suggested on the basis of several investigations (16, 17). Also, the requirement for DNA synthesis for release from mitotic inhibition has been demonstrated (18). Based on these findings, the existence of a repair cycle following irradiation and operating simultaneously with the normal cell cycle, is postulated.

With the results obtained for the cell age response of these cells a comparison is made between mitotic delay and cell killing. The survival data are in qualitative agreement with those obtained for L cells by other workers (13). However the mitotic delay results are not and, since no parallel sensitivity between the two effects is observed, the hypothesis of a common lesion (2) is thrown into further doubt. Further investigation is required in order to clarify the above discrepancies, and to more clearly identify the molecular bases for these phenomena.

II. MATERIALS AND METHODS

The Cell Line

A subline of L60T cells originally derived from Earle's fibroblastic L cells (19, 20) was used in all experiments. These cells are capable of growth on a glass surface or in suspension culture, and require no exogenous thymidine (TdR) or coenzymes in their growth medium. However, when TdR is administered to a culture, it is incorporated preferentially into the nucleic acid of the cells.

The cells were found to have a mean chromosome number of 51.5 ± 9.8 where the limits shown are the 95% confidence limits.

Growth Conditions

Cells to be used in the experimental procedures were maintained in suspension in glass spinner flasks[(a)]. The growth medium was CMRL 1066 (21) supplemented with 10% foetal calf serum,[(b)] but normally lacking coenzymes and thymidine. Antibiotics, pennicillin, streptomycin and anti PPLO agent[(b)] were added routinely to all cultures. Under these conditions the population doubling time was 15-21 hours. Cell growth was followed by making periodic cell counts with an electronic cell counter[(c)].

(a) Bellco Glass Inc., Vineland, New Jersey

(b) Grand Island Biological Co., Grand Island, New York

(c) Coulter Electronics Inc., Hialeah, Florida

Stock cultures were maintained in glass bottles kept at 37°C in a controlled atmosphere of humidified air and 5-10% carbon dioxide.

Irradiation Conditions

A 2000 curie Cesium-137 source was used for the irradiations. A diagram of this apparatus is shown in fig. 1. Radiation was delivered only when the lead-encased cylinder carrying the source was rotated to position A as shown. Samples to be irradiated were positioned to receive a dose rate of 100 rads per minute. Doses delivered were determined by a remote control timing device. Before and after delivery of the dose, the source was rotated to position B.

A Philips Universal dosimeter[(d)] was used to calibrate the apparatus, and a standardized Victoreen condenser chamber[(e)] fitted with a lucite, tissue-equivalent cap was used to correct the readings of the Philips dosimeter.

To insure a uniform dose delivered to the cells, the cultures were irradiated in suspension in spinner flasks.

It also was found necessary to maintain the cells at 37°C during irradiation, since a decrease of 1-2°C occasionally resulted in extended mitotic delays. A circulating water bath was used to maintain the temperature at the desired level during the irradiation.

(d) Philips Electronic Equipment Ltd., Toronto, Ontario

(e) Victoreen Nuclear Instrumentation, Cleveland, Ohio

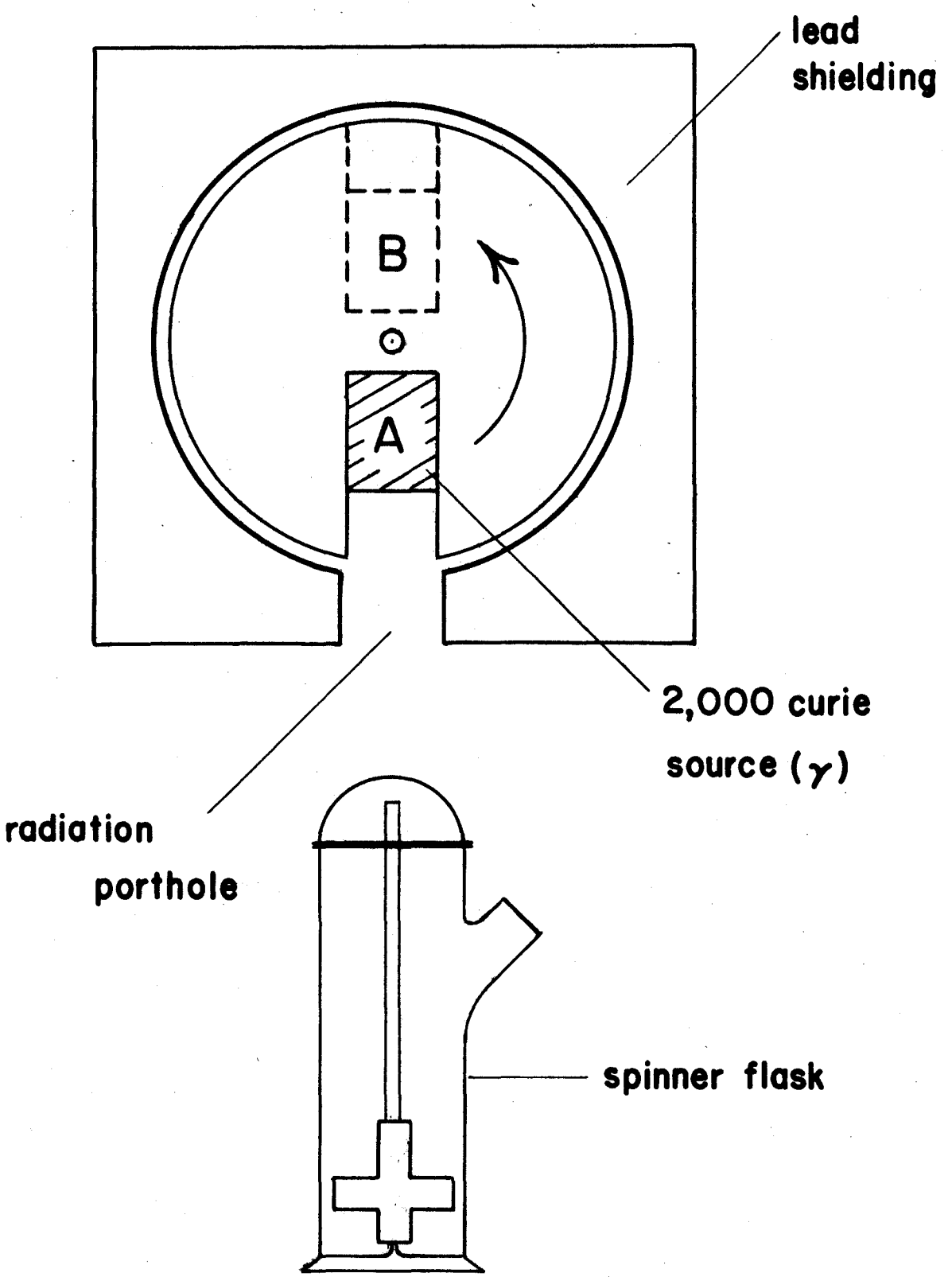

FIGURE 1. CESIUM-137 IRRADIATION APPARATUS

Cell Labelling and Autoradiography

Tritiated thymidine (H^3TdR) at specific activities of 6.7 c/mM or 17 c/mM[f] was added to exponentially growing suspension cultures at concentrations of 0.1, 1.0 or 2.0 μc/ml, depending on the experiment.

When it was desirable to label only the cells which were synthesizing DNA at a given time, a twenty minute pulse of H^3TdR was administered to the cells. Then the H^3TdR was diluted by 1/2800 or 1/1800 by dilutions of unlabeled TdR, which effectively stopped further significant labeling of the cells.

When a small, unlabeled segment of the cell cycle was desired, H^3TdR was administered to the culture and allowed to remain for a period which would allow all but a small portion of the cells to become labeled. Dilution of the H^3TdR was accomplished by centrifugation (600 rpm) at 37°C and resuspension in unlabeled medium conditioned by cell growth for 24 hours. Preconditioning of the diluting medium was necessary since resuspension in unconditioned medium caused variable and unpredictable cell growth. Alternatively a diluting concentration of unlabeled TdR was added at the appropriate time.

In some experiments continuous labeling of the cells was desired and in those cases the dilution step in the above procedure was omitted. The cells continued to be labeled as the population passed through the DNA synthetic phase of the cell cycle.

Cells from the labeled cultures were fixed at intervals in 3:1 ethanol-acetate and allowed to dry on microscope slides. The slide preparations were stained in 2% acetic-orcein and autoradiograms

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(f) New England Nuclear Corp., Boston, Mass.

were prepared by dipping the slides in Ilford G-5 emulsion[g] diluted 1/4 in distilled water at 45°C. Excess emulsion was allowed to drain from the slides. Then the slides were packed in opaque black slide boxes containing a dessicant, and stored at 4°C. After suitable exposure times, the slides were developed and examined microscopically for silver grains over the cells.

Cell Cycle Determination

The lengths of the various stages of the cell division cycle were determined using a pulse-labeling technique like that described by Defendi and Manson (22). The continuous labeling procedure of Stanners and Till (23) also was used.

In the first case, logarithmically growing cells were pulse-labeled with H^3TdR for 20 minutes during which time only the S phase cells were labelled. Following the pulse, hourly samples were taken from the culture and average grain counts over metaphases were obtained from the developed autoradiograms. Grain counts were made under oil immersion using a 100 x phase microscope objective.

In the second type of experiment, continuous labeling was used. Again, hourly samples were taken and autoradiograms prepared. Grain counts over metaphases and the percentage of unlabeled cells in the culture were determined. In this way it was possible to examine the flow of cells into the S and M phase.

Mitotic Inhibition

The procedure described by Whitmore *et al.* (13, 15) was used to obtain an unlabeled segment of the division cycle, a "window"

(g) Ilford Ltd., Essex, England

of cells which had not incorporated the label. H^3TdR was applied to an exponentially growing culture for 8-11 hours, depending on the growth rate of the cells. Dilution of the label after that time yielded a window of about 4 hours duration at the end of the G_1 phase.

Following the production of the window, the unlabeled cells composing it were allowed to move around the cell cycle to the stage under consideration. Then the population was split into small spinners preconditioned by cell growth for 24 hours. Again, the preconditioning was necessary to minimize the erratic cell growth observed when unconditioned glassware was used.

The small spinners were irradiated with a series of doses, most of which were large enough to produce mitotic delays longer than the time required for the unlabelled cells to reach mitosis if no irradiation had been given.

Colcemid(h) at 5×10^{-8} g/ml was added to each spinner shortly after irradiation, to accumulate cells as they reached metaphase. Samples were taken at two hour intervals, fixed and stained on slides, and the mitotic indices of the gross population were determined. Autoradiograms were prepared and were scored for the mitotic indices of the unlabeled cells.

Mitotic indices were determined from counts of 2000-5000 cells.

Loss of Proliferative Capacity

Puck et al. (24) have developed a convenient method to obtain survival curves for cells growing on glass. This procedure was used to obtain curves for the gross cell population.

(h) Courtesy of S. Mak, Queen's University, Kingston

After the cells were irradiated with a series of doses and diluted appropriately, they were inoculated into sterile glass bottles which had been seeded with heavily irradiated "feeder" cells the previous day. Puck has shown that the plating efficiency of cells is increased by the presence of such feeder cells in the growth medium. In these experiments 10^5 feeders per bottle were used. Four duplicate bottles were plated for each radiation dose.

After about 2 weeks' growth on glass, at which time the surviving cells had formed easily distinquishable clones, the cells were stained with methylene blue. The clones were counted and the fraction of cells surviving was determined.

The window technique already described for mitotic inhibition was used to determine variations in survival sensitivity around the cell cycle. Since it has been shown that incorporation of 1.0 μc/ml H^3TdR is lethal (25) to L cells, such a concentration was used in the production of a window of cells which had not incorporated the label. These cells were therefore the only viable cells in the population. The population was irradiated when the window had reached the stage of the division cycle under consideration, and appropriate dilutions of the irradiated cells were plated as described above. Radiation doses of 0-1200 rads were used, and survival curves were constructed.

III. RESULTS

Cell Cycle

As was described in the section on Materials and Methods, two procedures were used to determine the phases of the division cycle for the cell line.

In the first type of experiment, a pulse of H^3TdR was administered to an exponentially growing culture. Following the preparation of autoradiograms, grain counts over metaphases were made. The results of one such experiment are shown in fig. 2a.

Following the pulse, no silver grains appeared over metaphases until the labeled cells had moved through the G_2 phase. As the labeled cells reached mitosis, the grain counts increased to a maximum and, as these cells passed from mitosis the counts decreased again. The points of inflection in the curve of fig. 2a were chosen as the limits of the S phase since they reflect the maximum rates of increase and decrease of the label and, hence, should be representative of the majority of the cells in the population. To identify the points of inflection more accurately, the first derivative of the curve in fig. 2a was determined and is shown in fig. 2b. From the figure the values of G_2 and S are found to be 3.5 h and 5.8 h respectively.

As an estimate of the generation time, cell counts were made and a growth curve was plotted from which the doubling time (T_2)

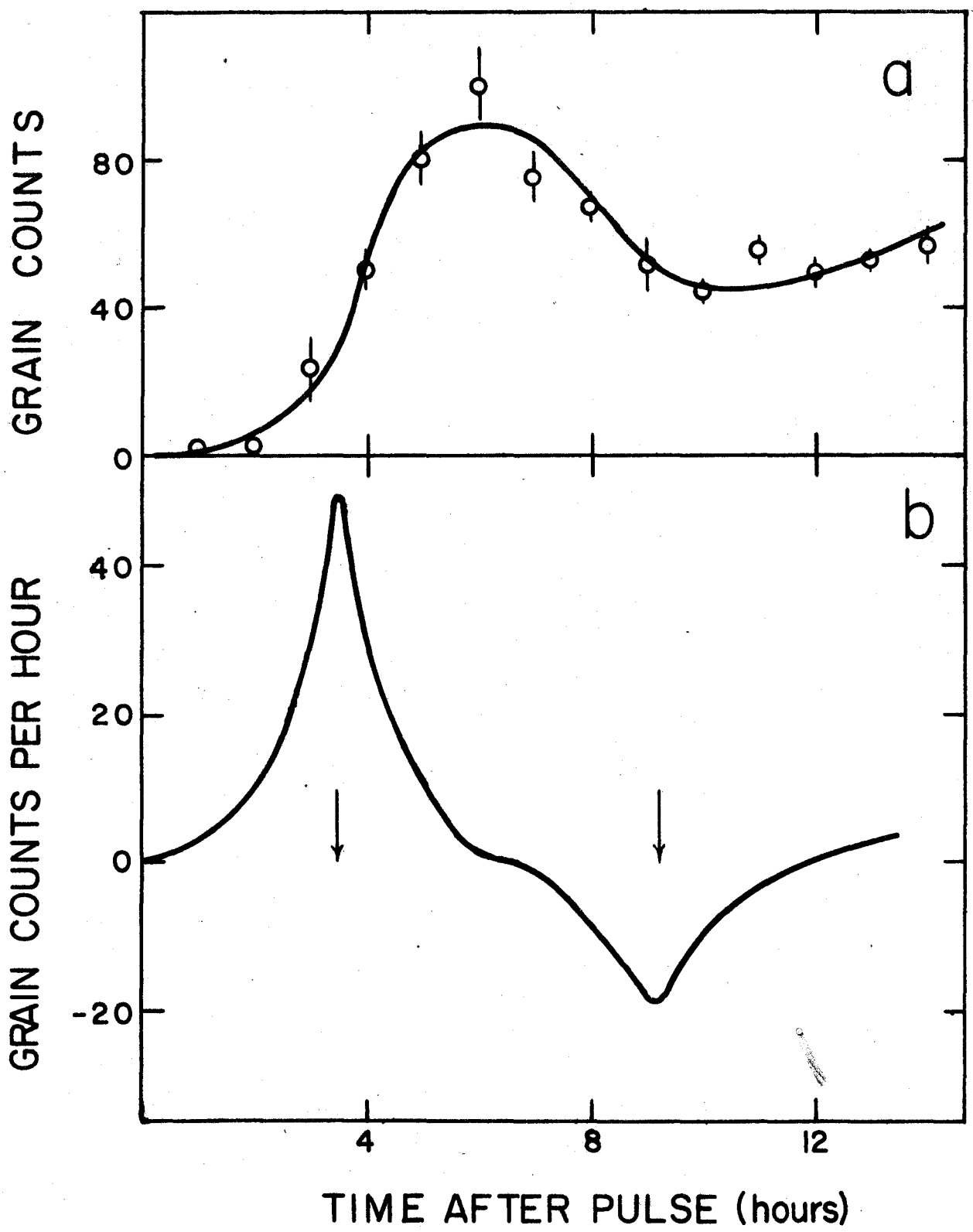

FIGURE 2. CELL CYCLE DETERMINATION, PULSE LABELING

of the population was determined to be 20 h. The growth curve is shown in fig. 3.

Mitotic indices were determined at hourly intervals for 20 hours from an exponentially growing culture. The mean value for the mitotic index was 1.8 ± .2 %. Again the limits are the 95% confidence limits. Since only a small variation in mitotic index with time was observed, a uniform distribution of cells around a 20 hour division cycle was assumed. The duration of the division cycle was estimated by the doubling time of the population.

For an asynchronous population in exponential growth, the length of the mitotic period cannot be obtained simply by multiplying the generation time by the fraction of cells in mitosis. Instead, account must be taken of the continual increase in the G_1 population as the cells pass around the cycle. From an equation developed by Stanners and Till (23) the duration of mitosis is given by

$$\tau_m = \frac{M.T}{0.693} - - - - (i)$$

where M is the fraction of cells in mitosis and T is the generation time. For the cells used in the present investigation, the duration of the mitotic phase is therefore $\tau_m = 0.52 \pm 0.05$ hours.

The length of the G_1 phase was found by subtraction to be 10.2 hours. The results of this experiment and another of the same type are shown under "pulse labelling" in table I.

As a check on the results of the pulse experiments, a second type of experiment was performed, using the accumulation of the tritium label to measure the flow of cells through the cycle.

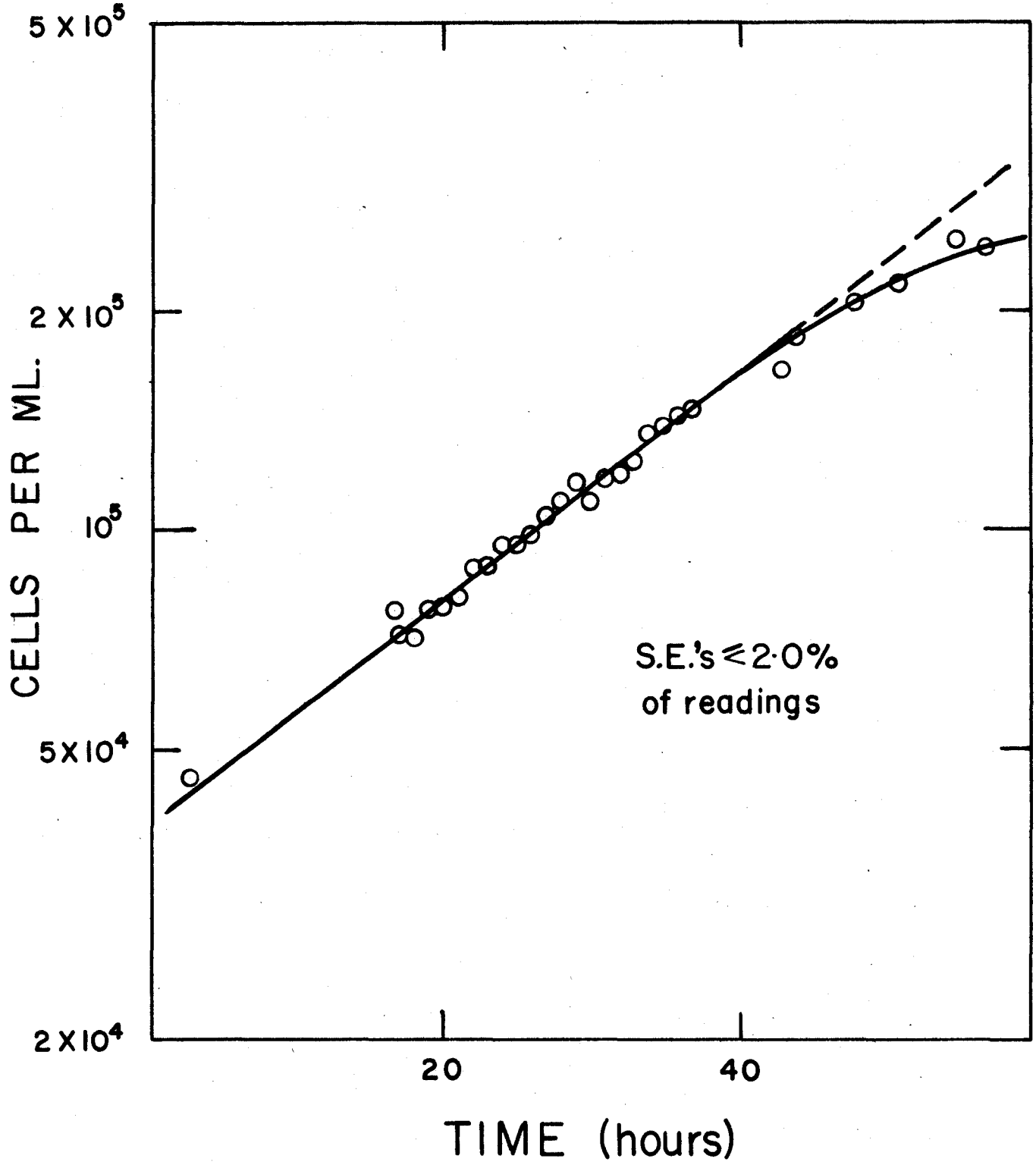

FIGURE 3. CELL GROWTH CURVE

TABLE I

LENGTH IN HOURS OF CELL CYCLE PHASES OBTAINED BY TWO METHODS

	H^3TdR LABELING PROCEDURE			
	PULSE		CONTINUOUS	
*T_2	20	14.5	19.5	19.5
*T_g	-	-	19.7	19.5
S	5.8	5.5	7.5	7.5
G_2	3.5	3.0	3.0	3.0
M	0.5	0.5	0.5	0.5
G_1	10.2	5.5	8.7	8.5

*T_2 is the population doubling time and T_g is the generation time obtained from labeling data.

H^3TdR was added to a growing culture and samples were taken at regular intervals. Again, grain counts over metaphases were made and growth curves were plotted. Also the percentage of unlabelled cells for each sample was determined. The results of one such experiment are shown in fig. 4.

As in the pulse experiments, following the addition of the label no grains appeared over metaphases until labeled cells had passed through G_2. Then the number of grains per metaphase increased for a time equal to the DNA synthetic period of the cells. After this period, cells reaching metaphase had been exposed to H^3TdR throughout their passage through S and therefore had accumulated the maximum amount of label. This is reflected in fig. 4 by a saturation in the grain counts over metaphases.

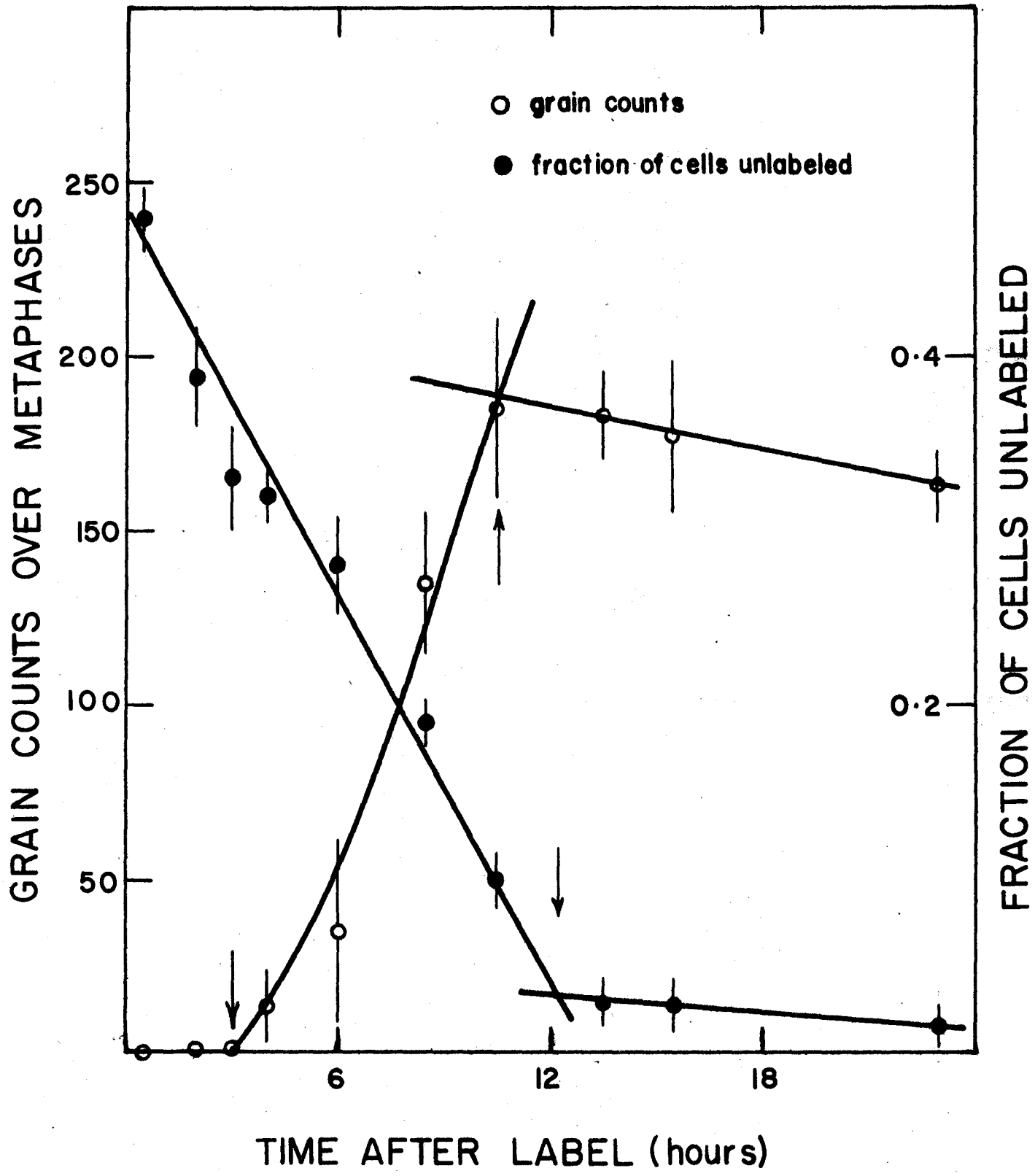

FIGURE 4. CELL CYCLE DETERMINATION, CONTINUOUS LABELING

Actually, in the saturation region of the curve, a decrease in grain counts was observed. The reason for this decay is not known conclusively, but an approximate calculation based on the amount of TdR required in 7 hours for DNA synthesis in these cells, suggests that the decrease might be accounted for by a depletion of the exogenous H^3TdR by those cells passing through S.

The intersection of the line drawn through the points occuring after saturation with that drawn through the increasing points prior to saturation marked the end of the S phase. The values for G_2 and S obtained from fig. 4 are 3.0 and 7.5 hours respectively.

In the same experiment, the percentage of unlabeled cells decreased with time until essentially all the cells were labeled. The small fraction remaining unlabeled probably represents cells which are growing very slowly or which are not proceeding around the cell cycle at all. Such non-proliferating cells may be said to be in a G_o state (26).

The time required for the maximum number of cells to become labeled corresponds to the total duration of $G_2 + M + G_1$ and in this case was 12.2 hours. Adding the value for S to this figure yields the generation time which is, therefore, obtained independently from the estimate based on the growth curve.

The results of this and one other experiment of this type also are shown in table I.

It can be seen that the estimation of S is different in the two procedures and that, in the experiment showing a population doubling time of 15.5 hours, the G_1 value is considerably smaller

than that obtained for a longer doubling time. This relationship between the length of G_1 and the doubling time has been confirmed by other workers. The discrepancy in S and in the $G_1 - T_2$ relationship will be discussed further in a subsequent section.

The combined results of the cell cycle determinations are depicted in fig. 5.

Mitotic Delay

Since variations in the generation time are reflected in the length of the G_1 phase of the cycle for this cell line, the doubling time of the population was determined prior to the addition of the label. On the basis of this determination, the length of time of exposure to the label was chosen to obtain a window of approximately 4 hours in each case.

The window, once produced, was allowed to move 0, 4 or 8 hours around the cycle before aliquots of the population were irradiated. Thus, at the time of irradiation the unlabeled cells were located in late G_1, S or G_2 respectively.

Mitotic indices were plotted as a function of time following the beginning of the irradiation. Typical mitotic inhibition release curves are shown in fig. 6. The effect of colcemid on the shape of the release curves can be seen by comparison with the results for cells receiving no colcemid.

Linear regression lines were drawn through the rapidly increasing portions of the inhibition release curves, and the points of intersection of these lines with the time axis were determined. The mitotic delay for each dose was defined as the time elapsed from the beginning of the irradiation to the point of intersection so determined.

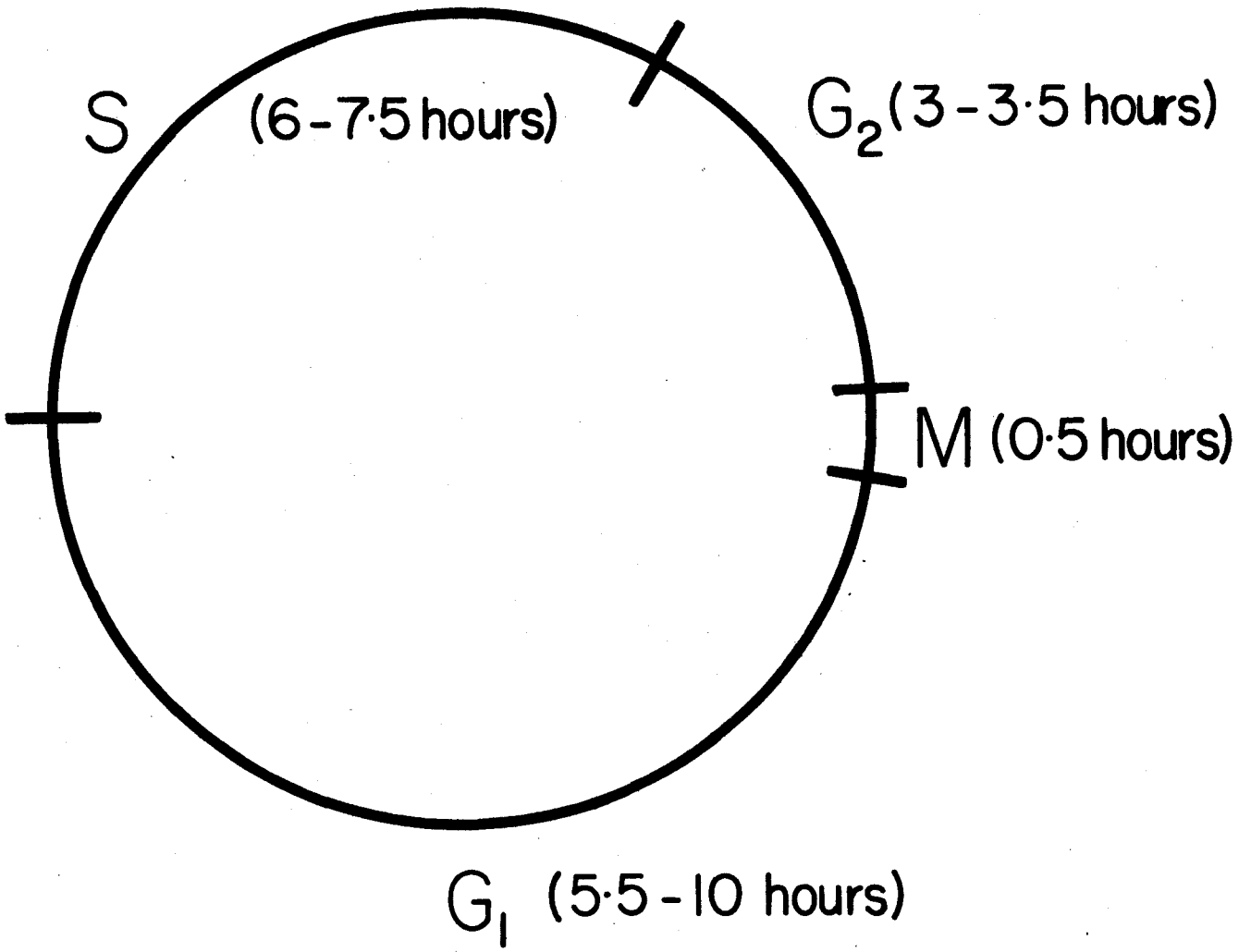

FIGURE 5. CELL CYCLE DIAGRAM

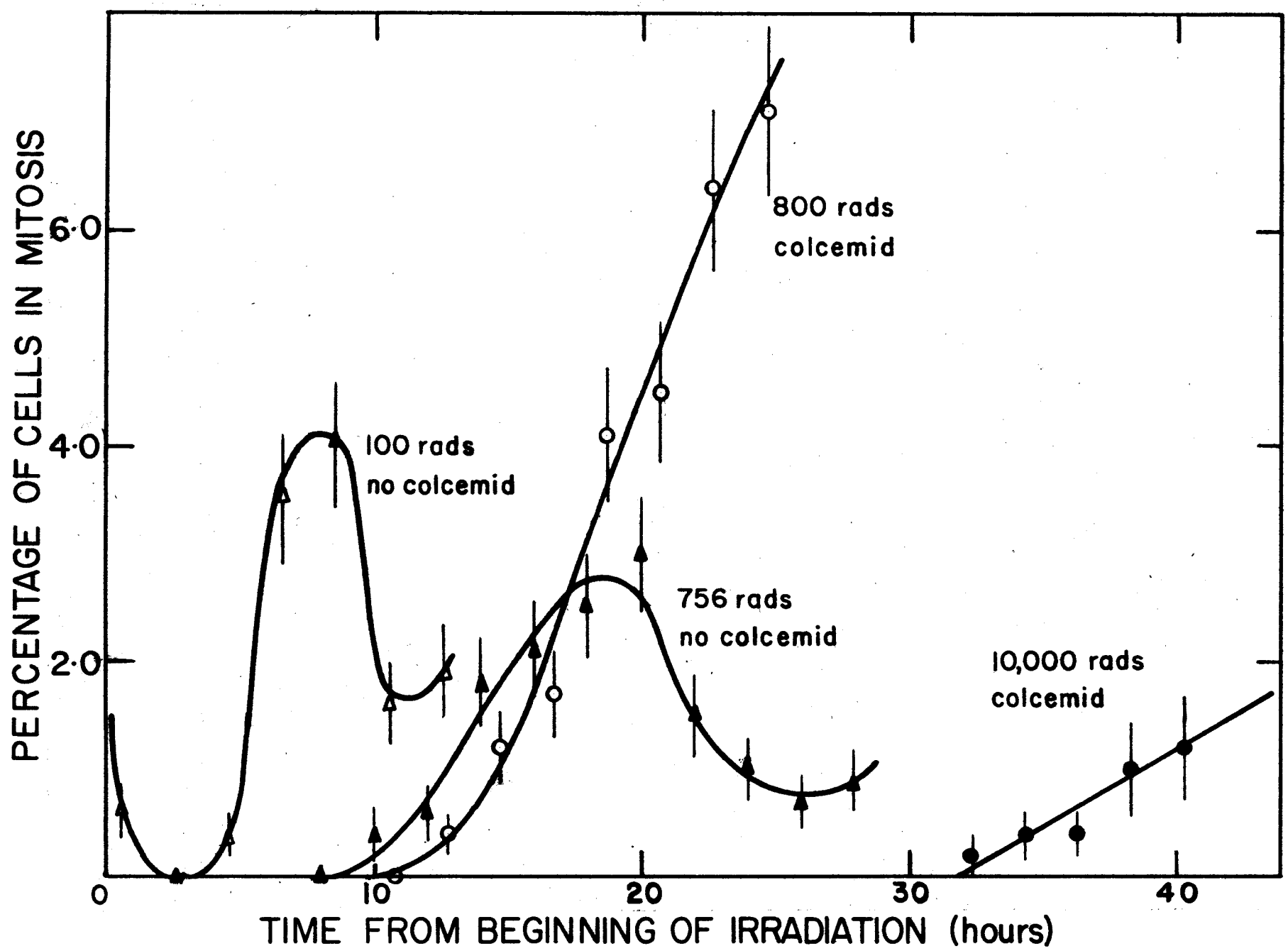

FIGURE 6. MITOTIC INHIBITION RELEASE CURVES

Following mitotic index determinations for the total cell population, autoradiograms were prepared and from them mitotic delay results for the unlabeled cells were obtained.

The mitotic delay t_L was plotted against the logarithm of the radiation dose D. The data for the gross population and for the unlabeled cells are plotted in this way in figs. 7, 8, 9 and 10. The limits shown are the standard errors calculated from the linear regression lines drawn through the inhibition release curves.

The straight line plotted in fig. 7 bears the slope of a curve recently published for our cells (27) and agrees closely with the data published by Whitmore et al. (15) for L60T cells. It is apparent, however, that this linearity does not apply over the large dose range used in this study.

The experimental points shown in figs. 8, 9 and 10 are the results obtained for unlabeled cells in G_1, S and G_2 respectively. The curve drawn in each case is identical to the curve drawn in fig. 7 for the gross cell population. Within the limits of experimental error, the data for cells in G_1, S and G_2 are superimposable on the curve for the total cell population. The S phase may appear to be more sensitive in the higher dose range, but the deviation seems negligible.

In general, these results do not agree with those reported by other workers (6, 12, 15) who found significant differences in the sensitivity to mitotic inhibition around the cell division cycle. A full discussion of these differences will be found in a subsequent section.

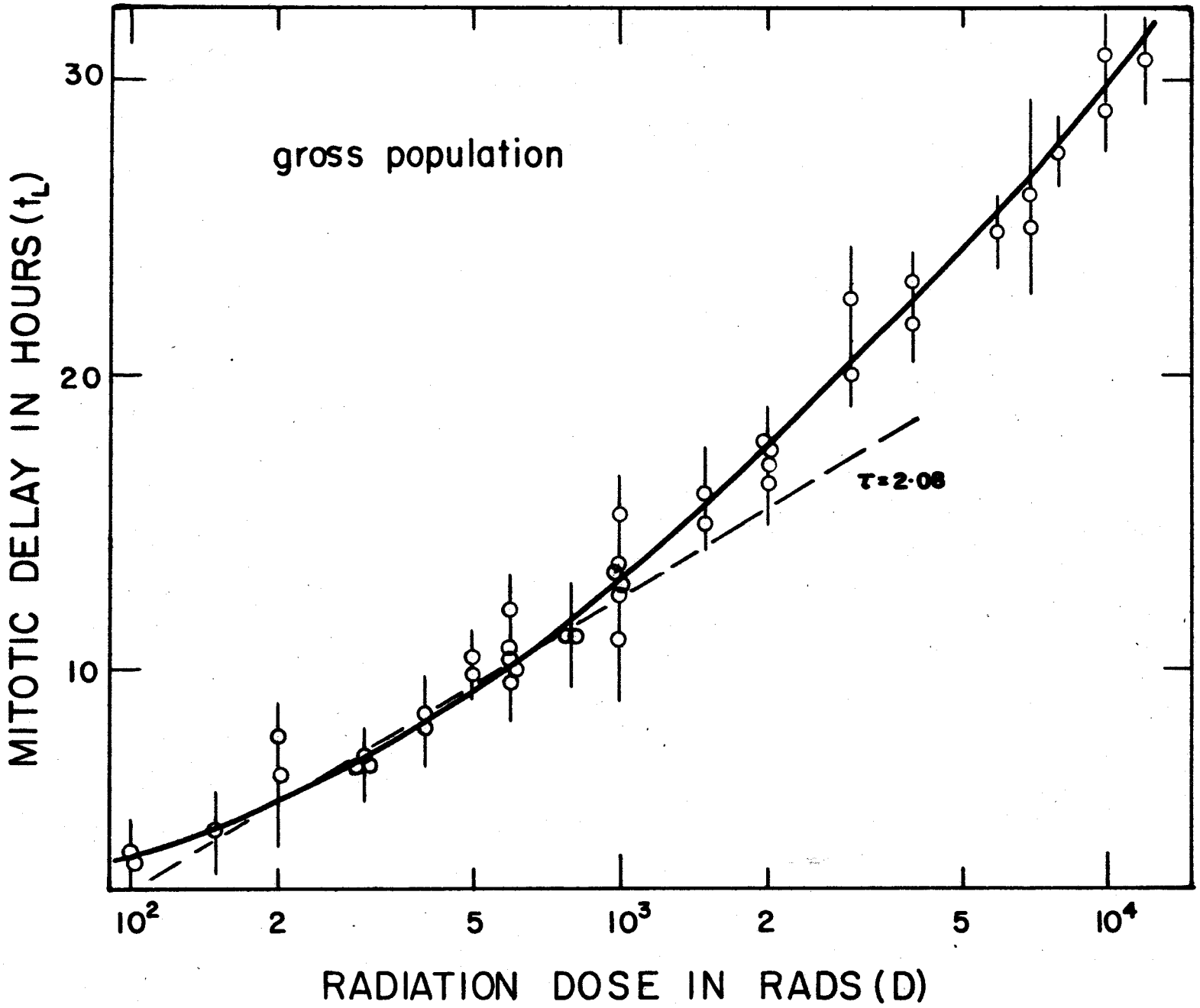

FIGURE 7. MITOTIC DELAY; GROSS POPULATION

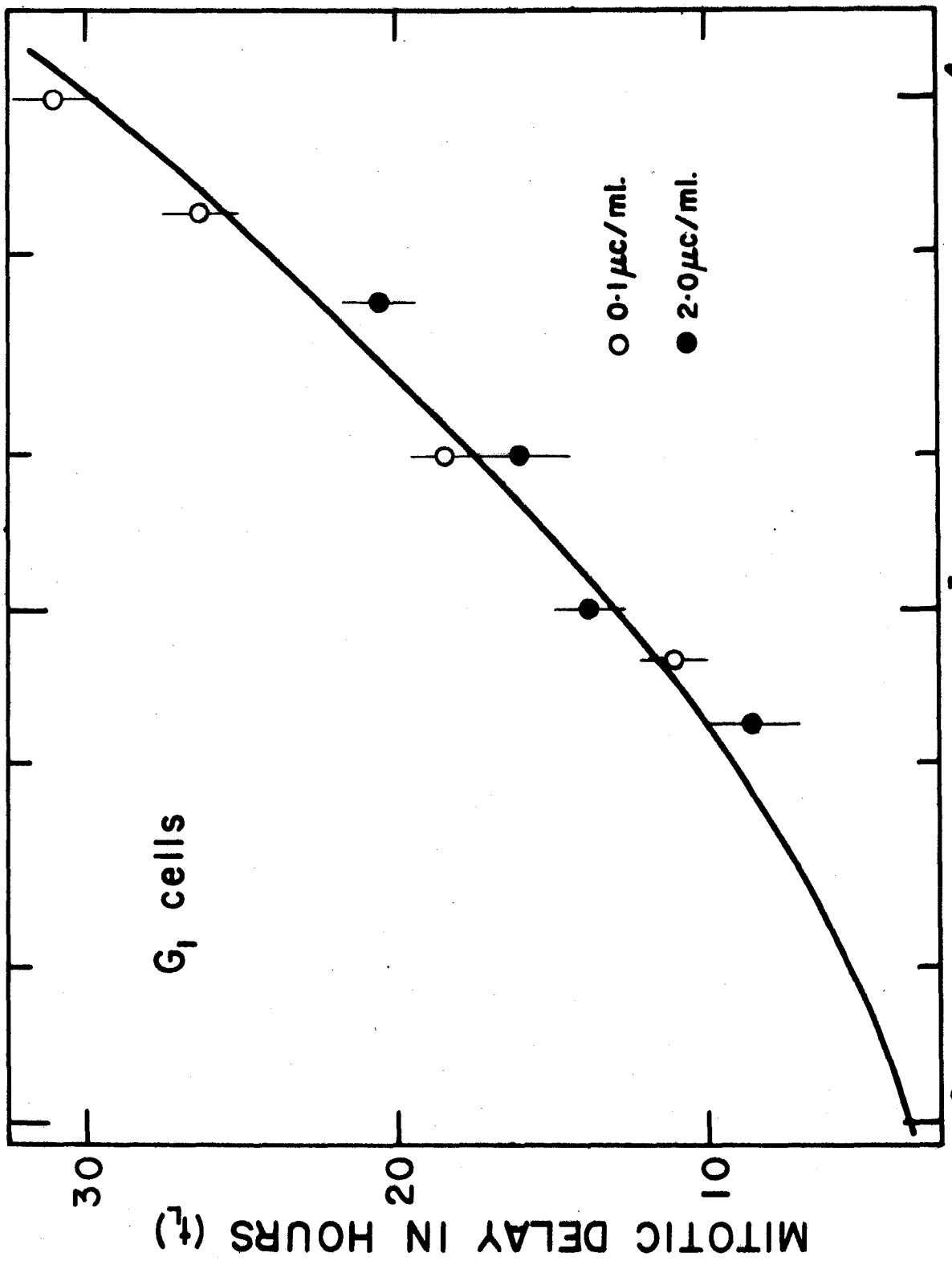

FIGURE 8. MITOTIC DELAY; G_1 CELLS

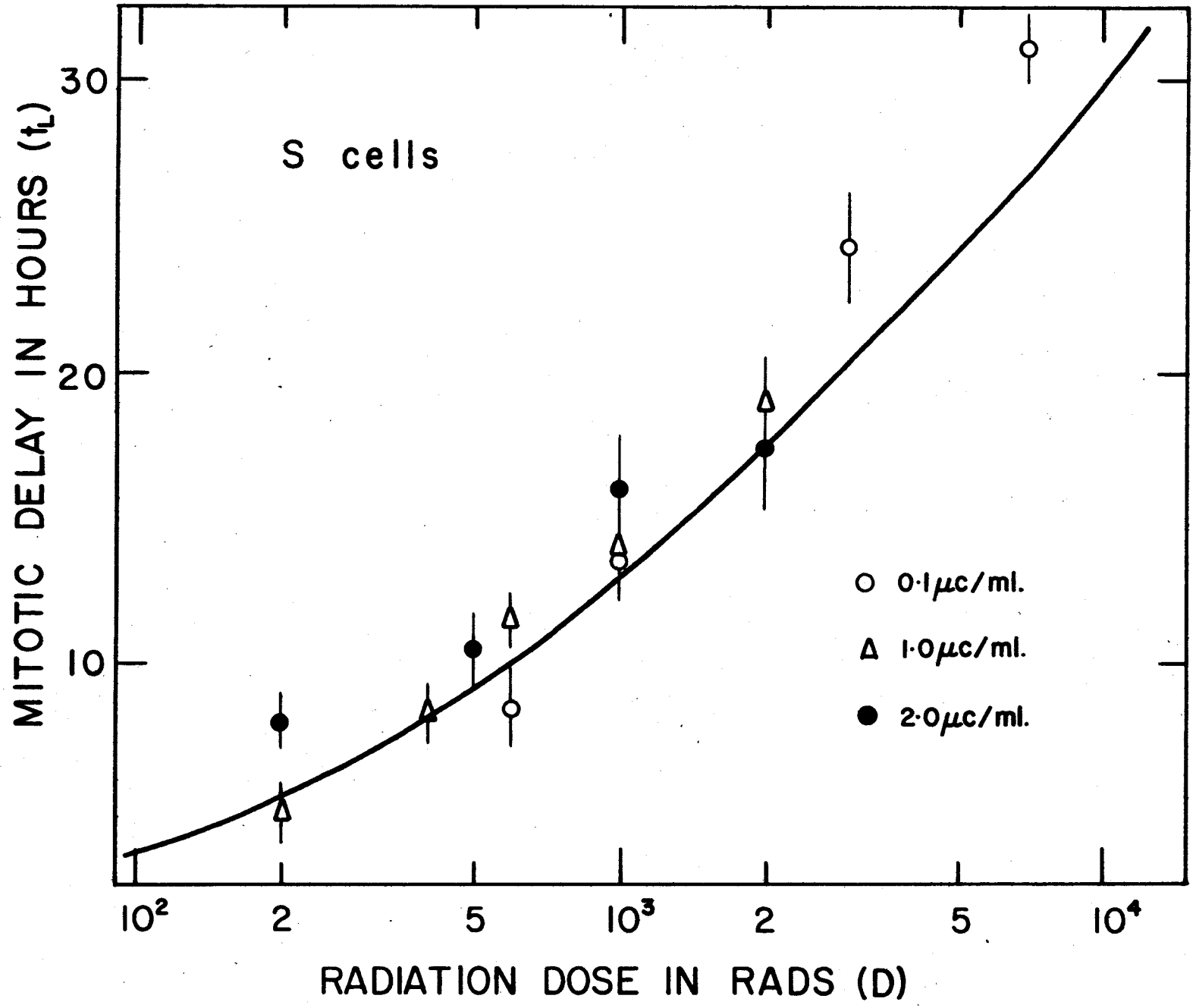

FIGURE 9. MITOTIC DELAY; S CELLS

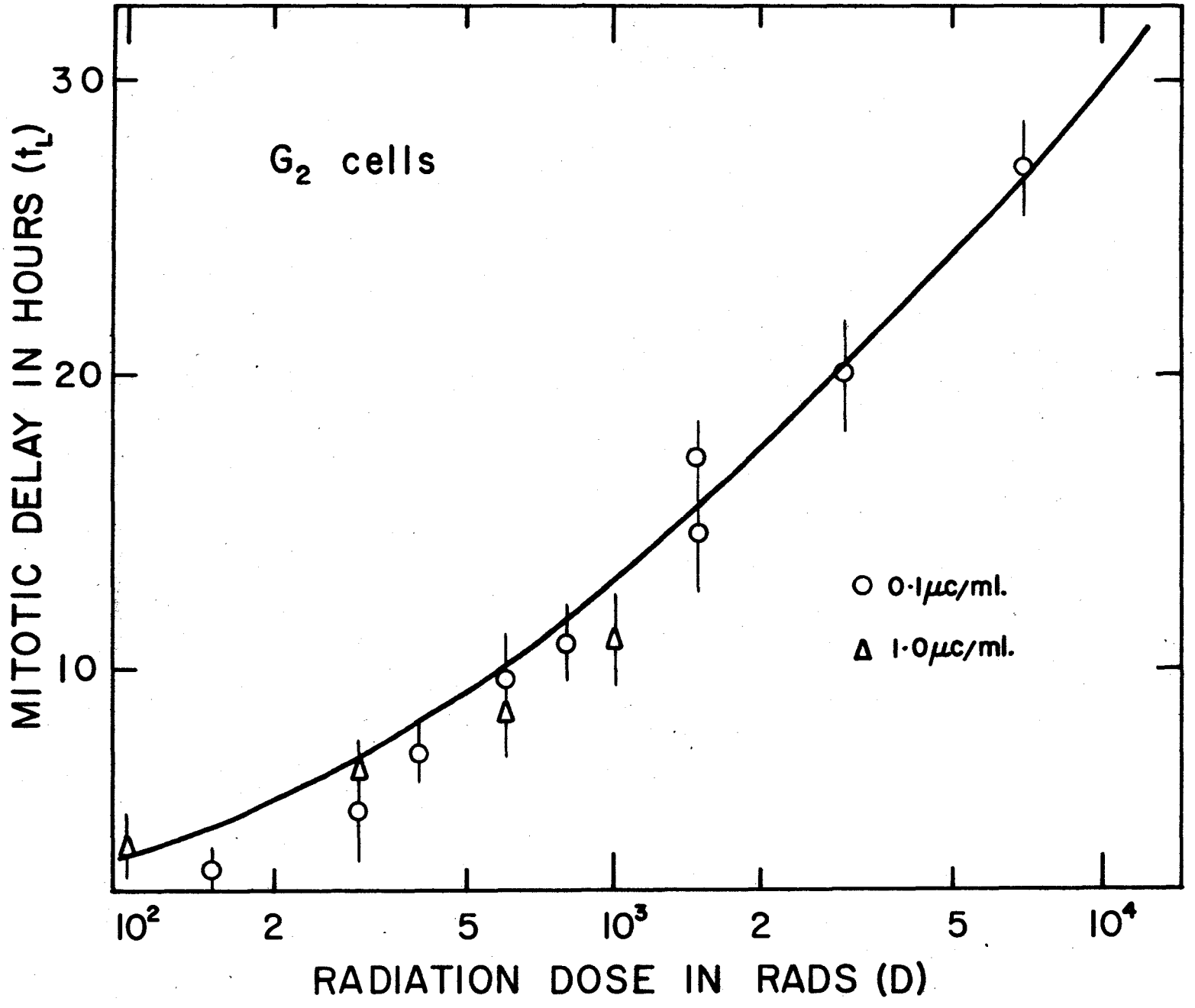

FIGURE 10. MITOTIC DELAY; G_2 CELLS

Loss of Proliferative Capacity

When the logarithm of the surviving fraction of a cell population is plotted against the dose, a survival curve consisting of a shoulder at low doses and a linear portion at higher doses is frequently obtained. Such curves can be characterized by two parameters, the D_o value which is the dose required to reduce the survival by 1/e on the exponential portion of the curve, and the extrapolation number n which is obtained by extrapolation of the exponential portion of the curve to zero dose.

Survival data were obtained for asynchronous cell populations and for cells in the various stages of the cell cycle. Insufficient data were obtained to determine values for n, but the D_o values are tabulated in table II. The limits shown are the 95% confidence limits.

TABLE II

VARIATION OF RADIATION LETHALITY AROUND THE CELL CYCLE

	Unlabeled population	G_1	S	G_2
D_o (rads)	207±9	242±16	210±23	178±20

The results are sufficient to indicate that the sensitivity of the cells to radiation lethality increases as the cells pass from G_1 to S to G_2. These findings are in qualitative agreement with the results of Whitmore *et al.* (13) for L cells.

IV. DISCUSSION

Cell Cycle

Two procedures were used to determine the length in time of the various stages of the division cycle. One method involved a pulse-labeling of the cells which were synthesizing DNA at the time of the labeling while the other employed a continuous labeling of the cell population as it passed through the S phase. The results indicate that, for this cell line, the cell cycle consists of a G_1 period of 5.5 - 10 hours followed by a DNA synthetic period (S) of 6 - 7.5 hours, a G_2 period of 3 - 3.5 hours and a mitotic phase of 0.5 hours. The experiments in which continuous labeling of the cells was employed yielded larger values for the duration of the S phase than the values provided by the pulse labeling procedure. This discrepancy can be accounted for by a further examination of the exact methods by which these values were obtained.

In the pulse-labeling experiments, curves of grain counts over metaphases versus time following the pulse were plotted. The points of inflection of these curves were determined as estimates of the beginning and end of the S phase. Since the points of inflection represent the maximum rates of increase and decrease of the labeling, this procedure is most likely to establish the S phase of the majority of the cells of the population.

The continuous labeling procedure, however, yielded consistently larger values for the S phase. Again, grain counts over metaphases

were plotted versus time. In any population of cells, there is expected a distribution of generation times as well as a distribution of the lengths of the various stages of the division cycle. In this case, therefore, the most rapidly cycling of the cells, located at the end of the S phase at the time of labeling, would be expected to be the first to contribute to the increase in grain counts over metaphases. Further, the cells with the largest generation times and longest S phases would have aided in an overestimation of S. Such cells located at the beginning of S at the time of labeling would have been the last to contribute to the increase in grain counts. The end of the S phase was determined by the point of saturation of the curve which therefore would have given an exaggerated value for the end of S. Hence, with the continuous labeling procedure it was possible to place an upper limit of 7.5 hours on the length of the S period for this cell line.

During these experiments an interdependence of the population doubling time and the length of the G_1 also was observed. The other phases of the cell cycle, however, remained relatively unaffected by changes in the doubling time. Since determinations of the generation time based on labeling data agreed very well with the doubling time of the population, it was assumed that the latter was a good estimate of the former.

The above relationship between generation time and G_1 confirms the reports of several workers (28, 29, 30, 31). Alteration of the generation time by temperature and pH change is found to be the result of changes in the length of G_1. Defendi and Manson

have attributed the variations in the generation times of different cell lines to differences in the duration of the G_1 stage. *In vivo* studies with the cells of normal and regenerating liver, and with normal and malignant cells, have demonstrated the same dependence.

Contrary evidence has been presented by Puck *et al.* (32) who showed that differences in generation time between HeLa and Chinese hamster cells were due to variations in the duration of all phases in the same proportion. Watanabe and Okada (16) have shown with mouse leukemic cells that, although G_1 is the most severely affected by temperature change, alteration in the generation time by temperature variation is also accompanied by significant changes in S.

The bulk of the evidence, however, points to a positive correlation between the length of G_1 and the cell generation time. The other phases of the division cycle remain relatively constant. Therefore it could be suggested that G_1 is involved in the synthesis of some compound essential to cell reproduction and that once this role of G_1 is completed, the cells proceed at the normal rate through S, G_2 and M. Moreover, since Dewey *et al.* (17) have demonstrated an increase in radiation-induced division delay with an increase in population doubling time, it could be suggested further that some function of the G_1 phase is involved in repair of the lesion responsible for mitotic inhibition.

Mitotic Inhibition

Following irradiation there is an immediate drop in the percentage of cells undergoing mitosis in a population of cells. After a time, which depends on the magnitude of the radiation dose,

there is a rapid increase in the mitotic index which is characteristic of the release of the population from mitotic inhibition. On the basis of the immediate decrease of mitotic activity on irradiation and the partial synchrony of the release curve, Whitmore et al. (33) have claimed the existence of a radiation-induced block located in late G_2 which temporarily prevents cells from progressing beyond that point. They found, moreover, that the progress of cells through G_1 and S was relatively unaffected.

However, Mak and Till (34) have reported that radiation doses of 580 - 10,900 rads delay the passage of L60T cells through all parts of the division cycle. In addition to the G_2 block, a temporary block was discovered in late S, and the rate of DNA synthesis was depressed. Passage of cells from G_1 to S was retarded also. Nevertheless, the G_2 block remained the largest of these post-irradiation effects.

From a comparitive study of a number of mammalian cell lines, Dewey et al. have shown that an increase in division delay is attendant on an increase in population doubling time. Furthermore, changes in the population doubling time are accompanied by changes in the length of the G_1 phase, as the discussion in the last section indicated. It might be suggested therefore, that some function of the G_1 phase is connected with the repair of the radiation-induced lesion responsible for mitotic inhibition.

Moreover, sensitization of L cells to mitotic delay by 5-bromodeoxyuridine (BUdR) has been recently demonstrated (27). BUdR, an analogue of thymidine, is incorporated specifically into the

nucleic acid of the cell. The observed sensitization implicates DNA in the radiation response. Experiments with 5-fluorodeoxyuridine (FUdR) confirm this finding (18). FUdR inhibits the enzyme thymidylate synthetase which is involved in the formation of thymidine. FUdR therefore interferes with the synthesis of DNA at this point. Treatment of the culture with FUdR prior to irradiation prevents the release from mitotic inhibition. Later addition of thymidine allows the FUdR block to be bypassed, and the cells may then divide. It would appear therefore that DNA synthesis is required for release from mitotic inhibition.

Since both DNA synthesis and a product of the G_1 phase appear to be necessary for the repair of the lesion responsible for mitotic inhibition, it is possible to suggest the existence of a "repair cycle" which operates parallel to and simultaneously with the normal cell division cycle. Consider, for example, a cell irradiated at some point in the cell cycle. The progress of the cell around the cycle continues until the G_2 block is reached. Some component required for further progress toward cell division is absent, the radiation either having destroyed it or having interfered with its synthesis. However, at the time of irradiation the repair cycle has begun to operate, and eventually the G_2 block is removed or avoided by the synthesis of the required substance. This repair cycle would require some functions of the G_1 phase, some functions of the S phase and perhaps some G_2 activity as well, though the latter is purely speculative.

Higher doses of radiation might be expected to interfere with a larger proportion of the normal metabolic processes. The reduced

rate of DNA synthesis and of cell passage into S as well as the block in S reported by Mak and Till might be explained on this basis. In addition the effectiveness of the repair system might be reduced at high doses.

Mitotic inhibition release curves yield different values for mitotic delay, depending on the criterion used to determine the time of release. In one investigation mitotic delay was taken as the time between irradiation and the time at which the mitotic index reached 25% of its average value in an unirradiated culture (27). Extrapolation to the time axis of the best smooth curve through the points yields comparable results. However, the objection to both of these is the difficulty encountered in the calculation of standard errors for mitotic delays.

In this investigation, extrapolation of a linear regression curve to zero mitotic index was used. This particular criterion is quite commonly used and it has the additional advantage of allowing convenient calculation of standard errors. Construction of these curves was facilitated by the use of colcemid, added to the cultures shortly after irradiation. Colcemid arrests the dividing cells quantitatively at metaphase and has no effect on the G_1, S, or G_2 phases (35). Cells thus are accumulated in metaphase as they pass through mitosis. Therefore, following release from mitotic inhibition the presence of colcemid insures a rapid increase in mitotic index with time. This is particularly important at very high doses where many of the cells are too greatly damaged to attempt mitosis, and where it is essential to detect the few cells that do divide in order to determine

the release curve. In this way the presence of the drug simplifies the drawing of the linear regression lines. Other evidence from this laboratory indicates that, although some concentrations of colcemid cause a prolongation of mitotic delay, the levels used in this investigation caused no significant increase.

Reports from a number of sources indicate that, when the mitotic delay is plotted against the logarithm of the dose, a straight line is obtained. This observation has been treated theoretically by Lea in his concept of cumulative dose (1). According to this model, the effective radiation damage to the cell at the end of the irradiation is due to a combined effect of continuous damage during irradiation and simultaneous exponential repair of that damage. This repair continues following irradiation and ultimately results in the release from mitotic inhibition.

The rate of decay of the cumulative dose (or damage) existing at time t is given by

$$\frac{dD}{dt} = I - \frac{D}{\tau} \quad - - - - \quad (ii)$$

where D = cumulative dose, I = dose rate, and τ = time constant for repair of damage.

If the dose rate is such that the irradiation time is negligible compared to the repair time, the equation may be approximated by

$$\frac{dD}{dt} = - \frac{D}{\tau} \quad - - - - \quad (iii)$$

which integrates to

$$\text{mitotic delay } t_L = - \tau \log D_L + \tau \log D_o - - - \quad \text{(iv)}$$

In this case D_o is the instantaneous damage sustained by the cell, and thus represents the radiation dose it has absorbed. The D_L term represents the level of damage at which no mitotic delay is observed. When the mitotic delay t_L is plotted against the logarithm of the dose D_o, a straight line should be obtained.

The experimental data were plotted in fig. 7 in accordance with this equation, the mitotic delays being measured from the beginning of the irradiation. As has been mentioned before, the results do not fall on a straight line.

However, since the dose rate approximation given in equation (iii) may not be valid at high doses, an exact solution to Lea's equation was developed and is presented in the appendix. The data were corrected appropriately and plotted as the delay from the end of irradiation ($t_L - t'$) versus the cumulative dose D' at the end of irradiation. The corrected points are plotted in fig. 11, with the curve from fig. 7 included for comparison. It is apparent that, even when the data are plotted according to an exact solution of Lea's equation, the relationship he has predicted does not fit the present data except, possibly, over a small dose range. The reason for the shape of the curve obtained in this study is not known, but it might be expected that for high doses (> 1000 rads) the components of the repair system itself may be damaged, causing a greater increase in delay with increasing dose.

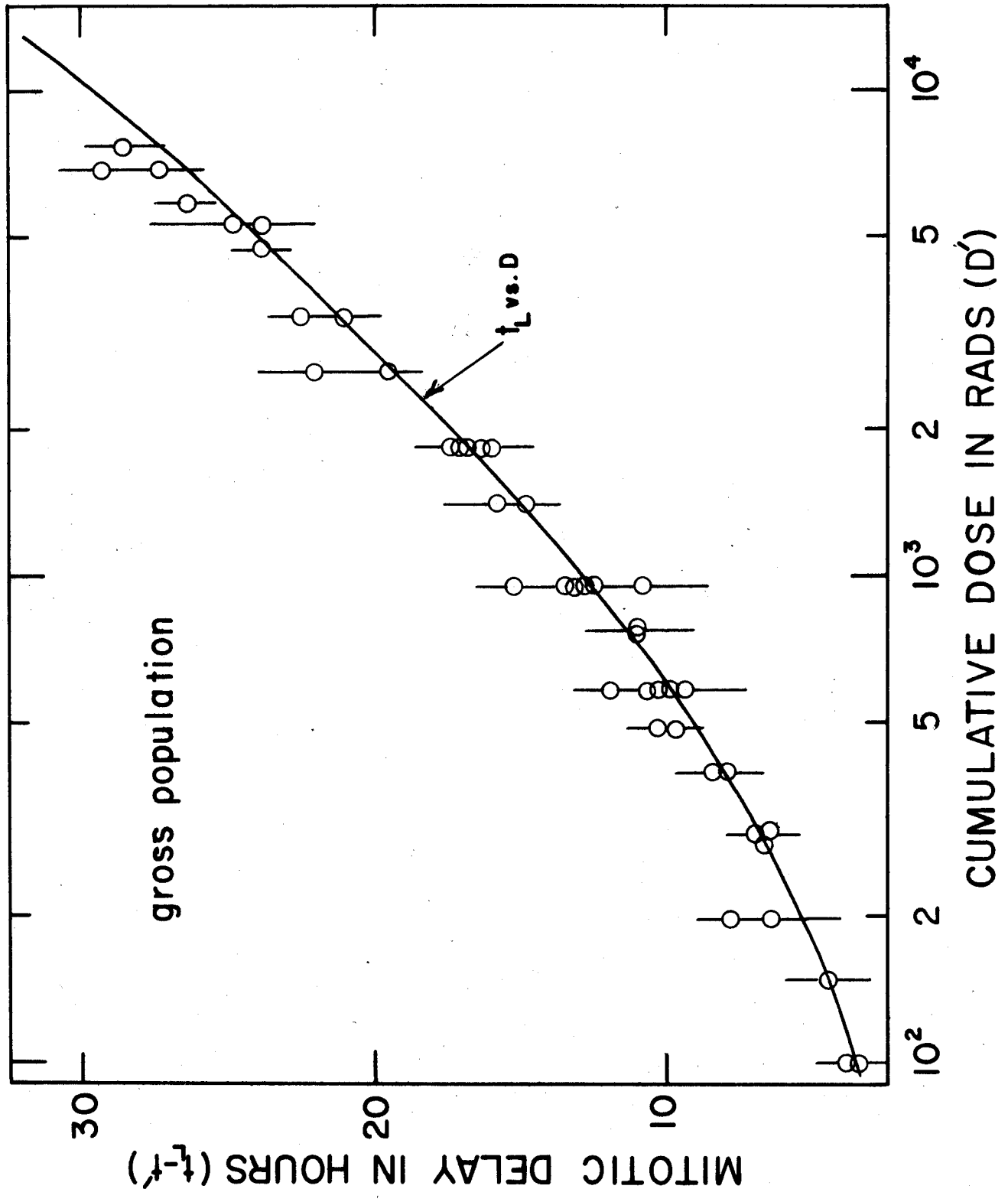

FIGURE 11. CORRECTED MITOTIC DELAY; GROSS POPULATION

Using tritium to label all but a small segment of a population of cells, it was possible to look at mitotic delays of cells irradiated at different stages of the division cycle. Mitotic inhibition release curves were obtained and the data were plotted in the manner of Lea described above. The results obtained for the various stages of the cell cycle are not significantly different from those just described for the gross population. All the curves demonstrated the same nonlinearity characteristic of the gross population.

In some experiments, lethal concentrations of H^3TdR were used in the production of the unlabeled window. The results for mitotic delay were the same when sublethal levels of tritium were used, indicating that the cells which had not incorporated the label were unaffected by the treatments. Removal of the label by low speed centrifugation and resuspension yielded data essentially identical to those obtained by dilution, showing that the concentrations of thymidine used in the dilution experiments had no detectable effect on the response of the cells to mitotic inhibition.

Other workers have examined the mitotic delay response to ionizing radiation as a function of the cells' position in the division cycle. Dewey and Humphrey (6) using a pulse labeling technique, have reported that L-P59 mouse fibroblasts increase in sensitivity to mitotic delay as they pass from G_1 to S to G_2. Also Terasima and Tolmach (12), using a selective harvesting of cells in mitosis to obtain synchronous cultures of HeLa cells, found that the sensitivity increased in the same way. However, since both these investigations were carried out using only a single dose of radiation, a true dose-response relationship was not available.

Whitmore et al. (15) have recently demonstrated results for L60T cells qualitatively similar to those reported by the above workers, and they have plotted complete dose-response curves for doses between 0 and 1100 rads. The tritium labeling technique used in the present investigation was employed.

The results reported here indicate no variation in sensitivity to radiation-induced mitotic delay as the cells pass through the division cycle, since the data for cells irradiated at various stages of the cycle are essentially superimposable on the data for the gross population. Therefore these results, at least below doses of 1100 rads, are in contradiction to the work of the above investigators.

Some of the differences, especially between these results and those of Dewey and Humphrey and of Terasima and Tolmach, may be attributed to differences in cell lines used. But there is probably much less difference between this cell line and that used by Whitmore et al., since our line was originally obtained from Whitmore's laboratory. Moreover, the mitotic delay data for the gross population are essentially identical for the two cell lines in the dose range used by Whitmore et al.

However, a discrepancy in the results of these workers is encountered when the data for cells in G_1, S and G_2 are combined to yield a composite mitotic delay curve for the gross population. Such combined results should approximate closely the actual data obtained for the total cell population. Considerable disagreement is evident, and might possibly be due to their particular experimental treatment of the cells. Speculation into the reasons for the differences between Whitmore's results and those reported here should await resolution of the above discrepancy.

Loss of Proliferative Capacity

The criterion usually employed to determine whether or not a cell has survived a given dose of radiation is based on its ability to make some arbitrary number of divisions in some arbitrary time (36). In this investigation the surviving cells were taken to be those that formed macroscopically visible colonies ($\sim$50 cells) in about two weeks. Many of these colonies contained giant cells which had lost their capacity to enter division but which continued to be metabolically active.

The survival curves showed the typical sigmoid shape characteristic of many cell systems, in that they were composed of an initial shoulder followed by a region of exponential decline. Such curves can be characterized by two parameters, the D_o which measures the slope of the exponential region on a semi-logarithmic plot and the extrapolation number n which measures the extent of the shoulder.

Survival curves were obtained for cells located in G_1, S and G_2 at the time of irradiation as well as for an unlabeled population. Doses from 0-1200 rads were used. It was observed that the D_o values were fairly consistent from one experiment to another whereas the values for the extrapolation number fluctuated greatly. Hence only the D_o values were used as an indication of the variation in sensitivity with cell age. The reason for the day to day displacement in the curves is not known. It was observed that the D_o values in every

case were higher than the values obtained by Whitmore et al. (13) but that the variation in D_o with position in the division cycle is qualitatively the same. Since the survival curves in the present study were only taken through 3 decades, it may be that the shoulder made a larger contribution to a determination of D_o than was justified. Since few experiments were performed, the confidence limits placed on the D_o values are rather large.

At any rate, the results of the survival experiments indicate that sensitivity to radiation lethality increases as the cells progress around the division cycle from G_1 to S to G_2. Although these results agree with those of Whitmore, investigations of other workers using other mammalian cell lines have yielded different conclusions. Terasima and Tolmach (12) have shown, for a single dose of 300 rads, that HeLa cells in mitosis and late G_1-early S are most sensitive to cell killing, those in G_1 and G_2 being the most resistant. Synchronization was achieved by selecting cells in mitosis. Sinclair and Morton (37) using the same method of synchronization showed that Chinese hamster cells exposed to a single dose of 750 rads were least sensitive when most of the cells were synthesizing DNA. The work of Dewey and Humphrey with the L-P59 cell strain indicated that, for a dose of 1500 rads, the G_1 cells were less sensitive than those in S, the G_2 cells being intermediate in sensitivity (38).

Undoubtedly, some of the discrepancies in the results of the above workers are due to inherent properties of the cell lines used, and may also be attributed to differences in experimental procedures. For these reasons it is probably only valid to compare the present results

with those of Whitmore et al. (13), since our cell line and experimental procedure were similar to theirs. In addition, the method employed in these experiments has permitted the construction of complete survival curves, which has not been done by the other workers.

The results of this investigation indicate that when L60T cells are irradiated at different stages of their division cycle, the dose-response curve for mitotic inhibition shows no variation from one stage to another, whereas the survival curves show decreasing values in D_o as the cells move from G_1 through S to G_2. The divergence in the sensitivities of these two effects adds further support to the suggestion (9, 10) that mitotic delay and loss of proliferative capacity are separate phenomena, and probably do not result from a common lesion, as Puck has claimed (2).

If indeed mitotic delay and cell killing are separate consequences of cell irradiation, it should be possible to further separate their effects and to examine each on a more fundamental level. Biochemical genetic studies of these effects should be possible. Radiation sensitivity studies with Escherichia coli strain B/r, first described by Witkin (39), have been extensive. The resistance of this strain to cell killing by ultraviolet light and X rays has been shown to be due to a single spontaneous mutation.

The advantages of microorganisms in such studies are considerable, since such cultures are genetically homogeneous and are capable of growth on simple, precisely defined media, neither of which descriptions apply to mammalian cells in vitro.

Davies, in his work with the unicellular green alga Chlamydomonas reinhardi, has described a differential sensitivity to cell killing by ultraviolet light as a function of cell position in the division cycle (40). This investigation has been extended further to similar studies with gamma rays (41), and significant variations in sensitivity were found. Dose fractionation experiments indicated that a recovery process was active, could occur at all stages, and was associated solely with the shoulder of the survival curve. The D_o values for a given phase remained constant while the extrapolation number varied. A strong similarity between the results of Davies and those of Elkind and Sutton (11) for mammalian cells, is evident.

C. reinhardi also would appear to be a good choice for further investigation into mitotic delay. Synchronous growth of an entire population is induced by exposure to light and this synchrony can be maintained for many generations. Cytological studies indicate that mitosis in this organism is normal (42) and extensive genetic maps are available (43). If the phenomenon of mitotic delay could be demonstrated in these cells, it should be possible to isolate mutants which have abnormally resistant or sensitive responses to the effect. Once such mutants were obtained, further biochemical studies might well make possible an identification of the repair process involved in mitotic inhibition.

APPENDIX

EXACT SOLUTION OF LEA'S EQUATION FOR MITOTIC DELAY

During irradiation, there are two processes operating. At dose rate I damage increases linearly with time from t_o to t', at which time the irradiation is stopped. However, exponential repair also begins at t_o. Thus at time t' the damage D will have been modified to some value D' by the repair.

During irradiation

$$\frac{dD}{dt} + \frac{D}{\tau} = I$$

$$D' = e^{-t'/\tau} \int_o^{t'} I\, e^{t/\tau}\, dt$$

$$= e^{-t'/\tau}\, I\tau \left(e^{t'/\tau} - 1 \right)$$

$$D' = I\tau \left(1 - e^{-t'/\tau}\right) - - - - (A)$$

following irradiation, I = 0, and exponential repair alone is operating

$$\frac{dD}{dt} = - \frac{D}{\tau}$$

$$\int_{t_L}^{t'} \frac{dt}{\tau} = - \int_{D_L}^{D'} \frac{dD}{D}$$

$$(t_L - t') = - \tau \log D_L + \tau \log D' - - - - (B)$$

Plotting $(t_L - t')$ against values of log D' from (A), a straight line is expected.

Correction of the experimental data in terms of these equations requires an experimentally determined value of τ. This was obtained from the slope of the straight line shown in fig. 7. This line passes through the low dose region of the curve where the dose-rate approximation given by equation (iii) in the text is valid, and yields a value of $\tau = 2.08$. The dose rate was $I = 6000$ rad/hr.

BIBLIOGRAPHY

1. D. E. Lea Actions of Radiations on Living Cells, Univ. Press, Cambridge, 1947.
2. T. T. Puck and M. Yamada Radiation Res. 16, 589, 1962.
3. J. F. Whitfield, R. H. Rixon and T. Youdale Exptl. Cell Res. 27, 143, 1962.
4. J. F. Whitfield and R. H. Rixon Exptl. Cell Res. 27, 154, 1962.
5. J. F. Whitfield, H. Brohee and T. Youdale Exptl. Cell Res. 35, 207, 1964.
6. W. C. Dewey and R. M. Humphrey Radiation Res. 16, 503, 1962.
7. W. C. Dewey and R. M. Humphrey in Cellular Radiation Biology, Williams and Wilkins, Baltimore, 1965.
8. T. T. Puck Revs. of Modern Phys. 31, 433, 1959.
9. L. D. Skarsgard in Radiation Effects in Physics, Chemistry and Biology, Ebert and Howard, North-Holland, Amsterdam, 1963.
10. D. O. Schneider and G. F. Whitmore Radiation Res. 18, 286, 1963.
11. M. M. Elkind and H. Sutton Radiation Res. 13, 556, 1960.
12. T. Terasima and L. J. Tolmach Biophys. J. 3, 11, 1963.
13. G. F. Whitmore, S. Gulyas and J. Botond in Cellular Radiation Biology, Williams and Wilkins, Baltimore, 1965.
14. A. Howard and S. R. Pelc Heredity (Suppl.) 6, 261, 1953.
15. G. F. Whitmore, J. E. Till and S. Gulyas Radiation Res. 30, 155, 1967.
16. I. Watanabe and S. Okada J. Cell Biology 32, 309, 1967.
17. W. C. Dewey, R. M. Humphrey and B. A. Jones Int. J. Rad. Biol. 8, 605, 1964.

18. I. M. Fettes, M.Sc. thesis, McMaster University, 1967.

19. K. K. Sanford, W. R. Earle and G. D. Likely J. Natl. Cancer Inst. 9, 229, 1948.

20. J. E. Till, G. F. Whitmore and S. Gulyas Biochim.Biophys. Acta 72, 277, 1963.

21. R. C. Parker in Methods of Tissue Culture, 3rd ed., Paul B. Hoeber, New York, 1961.

22. V. Defendi and L. A. Manson Nature 198, 359, 1963.

23. C. P. Stanners and J. E. Till Biochim. Biophys. Acta 37, 406, 1960.

24. T. T. Puck, D. Morkovin, P. I. Marcus and S. J. Ciecivra J. Exp. Med. 106, 485, 1957.

25. G. F. Whitmore and S. Gulyas Science 151, 691, 1966.

26. L. G. Lajtha Postgrad. Med. J. 38, 41, 1962.

27. D. O. Schneider and R. M. Johns Radiation Res. 28, 657, 1966.

28. J. E. Siaken and R. Kinosita J. Biophys. Biochem. Cytol. 9, 509, 1961.

29. V. Defendi and L. A. Manson Nature 198, 359, 1963.

30. D. B. Carter, B. E. Holmes and L. K. Mee Acta Radiol. 46, 655, 1956.

31. A. B. Reiskin and M. L. Mendelsohn Cancer Res. 24, 1131, 1964.

32. T. T. Puck, P. Sanders and D. Petersen Biophys. J. 4, 442, 1964.

33. G. F. Whitmore, C. P. Stanners, J. E. Till and S. Gulyas Biochim. Biophys. Acta 47, 66, 1961.

34. S. Mak and J. E. Till Radiation Res. 20, 600, 1963.

35. T. T. Puck and J. Steffen Biophys. J. 3, 379, 1963.

36. T. T. Puck and P. I. Marcus Proc. Natl. Acad. Sci. U. S. 41, 432, 1955.

37. Q. K. Sinclair and R. A. Morton Nature 199, 1158, 1963.

38. W. C. Dewey and R. M. Humphrey Nature 198, 1063, 1963.

39. E. M. Witkin Genetics 32, 221, 1947.

40. D. R. Davies Mutation Res. 2, 477, 1965.

41. D. R. Davies Third Internatl. Congress of Radiation Res., 1966.

42. N. D. Buffaloe Bull. Torr. Bot. Club 85, 157, 1958.

43. W. T. Ebersold, R. P. Levine, E. E. Levine and M. A. Olmstead Genetics 47, 531, 1962.