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RELEASE OF

RADIATION-INDUCED MITOTIC INHIBTION

IN MAMMALIAN CELLS

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IN MAMMALIAN CELLS

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SCOPE AND CONTENTS: The requirement of DNA synthesis for the release of Y-radiation-induced mitotic inhibition in mammalian cells has been studied. Mammalian cells in which DNA synthesis had been inhibited by treatment with fluorodeoxyuridine (FUdR) were not released from radiation-induced mitotic inhibition until the FUdR block was removed. After removal of the block, mitotic figures reappeared, but only after a time equivalent to the usual mitotic delay caused by the particular radiation dose employed. This suggests that repair of the mitotic inhibition lesion can not proceed unless the pathway for DNA synthesis is intact. Further evidence for the requirement of DNA synthesis in the release of mitotic inhibition came from the observation of radiation-induced synthesis of DNA during G_2, a stage in the cell cycle normally not associated with such synthesis.

PREFACE

This thesis describes work carried out in the Research Unit in Biochemistry, Biophysics and Molecular Biology from October 1965 to September 1967.

The author wishes to express her sincere appreciation to Dr. D. O. Schneider for the supervision of this project.

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Research Unit in Biochemistry,
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INTRODUCTION

Increasing interest in the use of ionizing radiation in medicine and in industry and the widespread awareness of possible radiation hazards in the environment have provided a great impetus for combining the study of molecular biology and ionizing radiation. In addition to the more practical value of studying the effects of radiation on living systems, one can use such investigations to achieve a better understanding of cell structure and function. In recent years there has been particular interest in using radiation techniques to study the operative control mechanisms in living cells. Of concern in the present investigation is the cellular control mechanism for regulating mitotic activity.

One of the effects observed after a population of mammalian cells has been exposed to ionizing radiation is the immediate, temporary disappearance of mitoses from the cell population. This phenomenon is called mitotic inhibition and the time duration between irradiation and the reappearance of mitoses is the mitotic delay. The present investigation was carried out in an effort to determine if deoxyribonucleic acid (DNA) synthesis is required for the release of mitotic inhibition.

Other cytological phenomena observed subsequent to the irradiation of mammalian cells are chromosomal aberrations and cell death. Correlations have been drawn between prolonged chromosomal elongation and mitotic delay (1, 2, 3) and chromosomal aberrations and cell death (4, 5, 6) and in fact Puck (7) has suggested that

mitotic delay, chromosomal oberrations and cell death are caused by the same chromosomal lesion. However, the work of Schneider and Whitmore (8) and Skarsgard (9) on the relative biological efficiencies (R.B.E.) of radiations of various linear energy transfers (L.E.T.) strongly indicates that different mechanisms are responsible for cell death and mitotic delay. It seems probable that the lesion which is responsible for cell death is chromosomal in nature, but it does not appear to be the same lesion which causes mitotic inhibition. However, the lesion giving rise to mitotic inhibition may indeed be chromosomal as well.

Repair of sublethal radiation damage was first demonstrated in the survival studies of Elkind and Sutton (10) on Chinese hamster cells. They performed a series of fractionation experiments and found that some radiation damage was repaired between doses. Whitmore et al. (11) claim that the degree of recovery observed between exposures is actually due to a combination of intracellular repair and progression of surviving cells around the cell cycle through phases of varying radiation sensitivity. Such phases of varying radiosensitivity have been found for a number of organisms (12, 13, 14) and attempts have been made to relate the sensitivities to G_1, S, G_2 and M, the four stages in the division cycle[(a)].

(a) The mammalian cell cycle has been divided into four stages (15): a mitotic period (M) during which cell division takes place, followed by G_1, a pre-DNA synthesis period, S, a period during which DNA is synthesized and finally G_2, a pre-mitotic, post-DNA synthesis period.

No consistent relationship, however, has been found for cell radiation survival. Investigations by Whitmore et al. (12) indicate an increase in sensitivity to mitotic inhibition as the cell progresses from G_1 to S to G_2. However, investigations carried out in this laboratory (16) indicate no significant variation in sensitivity to mitotic inhibition around the cell cycle.

The effects of radiation on DNA synthesis have been studied by several workers on a wide variety of cellular systems. An early study conducted by Euler and Hevesy (17) demonstrated that radiation reduced the uptake of radioactive phosphorus (P^{32}) into the DNA of Jensen rat sarcoma cells. Whitmore et al. (18) found close to normal rates of DNA synthesis during mitotic inhibition in asynchronous mouse L cells, followed by continued synthesis of DNA at a reduced rate after the release of mitotic inhibition. In E. coli X-ray doses of 2000 R or higher which have little effect on net DNA synthesis do seem to alter the normal sequence of replication of the bacterial chromosome (19). Radiation-induced DNA synthesis has been shown by increased incorporation of tritiated thymidine (H^3TdR) into the DNA of onion root tip cells (20), slime mold (21) and grasshopper neuroblasts (13). In the case of the grasshopper neuroblasts the unscheduled DNA synthesis took place at times in the cell cycle which are normally not associated with DNA synthesis. McGrath (13, 22) et al. think that the period during which X-ray-induced incorporation of H^3TdR can occur may also be the period when repair of radiation damage occurs.

Post-irradiation treatments with enzymatically hydrolyzed DNA as well as deoxyribonucleotides increase the survival of mouse L cells (23).

A similar effect had been obtained previously with highly polymerized isologous DNA. This is in agreement with the observed increase in survival of irradiated bacteria (24) and rats, and with restitution in some tissues (25) upon treatment with highly polymerized homologous DNA. On the contrary, post-irradiation treatment of HeLa cells with fluorodeoxyuridine (FUdR) or hydroxyurea, both of which act by inhibiting DNA synthesis (26, 27), enhances X-ray killing (28).

Pre-irradiation treatment of HeLa cells with FUdR or hydroxyurea apparently has no influence on the survival of G_1-cells if the inhibitory action is removed immediately after irradiation (28). Sinclair recently has demonstrated that X-ray survival of G_1 Chinese hamster cells, which had been inhibited from synthesizing DNA by treatment with hydroxyurea or excess thymidine, first declined and then increased steadily (29).

Although most data for cell survival generally seem to indicate that DNA is involved in repair of radiation damage it is not yet clear whether this involvement includes DNA synthesis. Elkind et al. (30) have proposed that the repair may be a nonenzymatic process involving DNA in a stereochemical relationship to its molecular environment. Their evidence suggests that radiation damage involves a disruption of DNA and that repair is a return of the relevant molecules to a functional state.

Much less is known about the involvement of cell DNA in the repair of the lesion inducing mitotic inhibition. That such a repair mechanism exists is implied by the very nature of the inhibition phenomenon. Mitotic delay has been described as the time duration

between irradiation and the reappearance of mitoses. Since mitoses do eventually reappear in the population, there must be some repair mechanism in operation. Lea assumed the existence of an exponential repair process in his theoretical treatment of mitotic delay (31).

That cell DNA is somehow involved in mitotic inhibition has been indicated by the increased radiosensitivity of mouse L cells by 5-bromodeoxyuridine (BUdR) (32). BUdR is an analogue of thymidine and has been shown to be incorporated specifically into the DNA of mammalian cells (33). Such incorporation causes a prolongation of mitotic delay following exposure to γ-radiation.

In this treatise, evidence for the requirement of DNA synthesis for the release of mitotic inhibition is presented. Mammalian cells in which DNA synthesis had been inhibited by treatment with FUdR were not released from the mitotic inhibition following irradiation until the FUdR block was removed by the addition of exogenous thymidine. Moreover, a slight incorporation of H^3TdR was observed in mouse L cells which were in G_2 at the time of irradiation. That is, a radiation-induced unscheduled synthesis of DNA was observed. The evidence suggests not only that DNA synthesis is required for repair of the mitotic inhibition lesion, but that it is probable that no repair at all can occur unless the pathway for DNA synthesis is intact.

CHAPTER I

MATERIALS AND METHODS

Cell Lines

Most of the studies were performed on a single line of mouse cells. Supporting evidence came from work on two other mammalian cell lines: a human cell line and a hamster cell line. All of the cell lines used can be grown in thymidineless medium, making them very suitable for the present investigation.

L-4 cells. The subline of mouse cells which was used is a derivative of Earle's original fibroblast line (34, 35). These cells were found to have a mean chromosome number of 51 ± 9.8, where the limits shown are the 95% confidence limits. In this respect, therefore, the L-4 line is not significantly different from "L" line mouse cells used in many other investigations. L-4 cells can be grown very readily either in suspension cultures or attached to glass surfaces.

H.Ep.-2 cells. The human tissue culture line used was originally isolated by Fjelde (36) from an epidermoid carcinoma of the larynx. The mean chromosome number in the H.Ep.-2 line is 77, with a variation in chromosome numbers between 69 and 81 (37). These cells also can be grown both in suspension and on glass surfaces.

H+ cells. The H+ cells are a subline of the H2 cells of Schneider and Whitmore (8), which were derived from the Chinese hamster embryonic fibroblasts of Yerganian and Ford (38). The mean chromosome number of

the H+ cells was found to be 24 ± 12.3, where the limits shown are the 95% confidence limits. These particular cells could not be grown in suspension and all experiments carried out with them involved cultures grown on glass.

Growth Conditions

All cells were grown at 37°C. For the cultures which were grown on glass, Brockway bottles(b) were used exclusively. For such glass-grown mammalian cell cultures, especially during the initial stage of low cell density, it is necessary to maintain them at a relatively constant pH. This was accomplished by growing the cells in a controlled atmosphere of humidified air and 5-10% CO_2. After a sufficient cell density has been reached, such cultures are able to self-maintain a suitable pH for growth and further control is unnecessary. However, for the experiments with glass-grown cells discussed in this thesis, external control of the pH of the growth medium was maintained throughout.

Most of the experiments involved cultures of cells in suspension, and spinner flasks(c) were used for these. For cells to grow in suspension it is necessary to have a concentration of at least 10^4 cells per millilitre. At such a concentration, the cells themselves are able to maintain a suitable pH for growth. As a result, control of the atmosphere was not necessary and the spinner flasks were kept tightly closed.

All cells were maintained in the logarithmic phase of growth through routine sub-culturing in fresh medium. Growth of cells in

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(b) Brockway Glass Co., Inc., Brockway, Pennsylvania

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

spinner flasks was followed by taking periodic counts with an electronic cell counter[d].

L-4 cells were grown both in suspension and on glass. Normally the growth medium consisted of C.M.R.L. 1066 (39) minus thymidine and coenzymes, but with antibiotics streptomycin, penicillin and anti-P.P.L.O. agent[e] added. Occassionally Eagle's minimal essential medium (40) was used in place of C.M.R.L. 1066 for some of the glass-grown stock cultures. All media were supplemented with fetal calf serum[e]. Although levels of 5%, 10% and 20% serum were originally attempted, the 10% level yielded the most satisfactory cell growth and was used throughout the L-4 cell experiments presented in this treatise. Because it was thought that the fetal calf serum might have contained a low level of thymidine, attempts were made to grow cells in serum which had been dialyzed. Unfortunately, it was found that serum which had been dialyzed for thirty hours in doubly distilled water would not support growth either on glass or in suspension. Substitution of phosphate buffered saline (PBS) (39) for the distilled water as a dialyzing bath resulted in serum which supported growth on glass indefinitely and growth in suspension for short periods of time. Because of the difficulty in growing suspension cultures in medium supplemented with dialyzed serum, such serum was used for only a few of the experiments. The doubling time of L-4 cells grown in suspension in medium supplemented with 10% dialyzed or undialyzed fetal calf serum varied from 16-26 hours. The doubling time of L-4

(d) Coulter Electronics Inc., Hialeah, Florida.

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

cells grown on glass was somewhat shorter, ranging from 14-18 hours.

The H.Ep.-2 cells also were grown both on glass and in suspension. The cells grown on glass were maintained in Eagle's minimal essential medium (M.E.M.). For H.Ep.-2 suspension cultures it is necessary to use a modified M.E.M. to prevent the cells from sticking to the glass surfaces of the spinner flasks. In this case Joklik's modification of M.E.M., which has 10X the phosphate concentration but lacks the $CaCl_2$ component, was used. The medium was supplemented with antibiotics and 10% undialyzed fetal calf serum. Again it was found that a level of 5% serum did not support growth adequately. The doubling time of the H.Ep.-2 cells varied from 17-25 hours.

The H+ cells were grown on glass in C.M.R.L. 1066 medium with added antibiotics, but lacking thymidine and coenzymes. For these cells, a 2½% level of undialyzed fetal calf serum was used during the experiments, while the stock cultures were maintained in medium supplemented with 10% fetal calf serum.

Irradiation Procedure

A 2000 curie cesium-137 source was used for all irradiations. Such a source produces gamma rays of 660 KeV energy, but after scattering and absorption in the source itself, some lower energy components are present in the radiation beam.

Cell doses were delivered at a rate of 100 rads per minute. Dose measurements were made with a Philips Universal Dosimeter(f)

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

which, in turn, was calibrated against a standardized Victoreen condenser chamber[g].

The L-4 and H.Ep.-2 cells were irradiated in suspension in the spinner flasks in which they were grown. During the irradiation, the cells were maintained at 37°C by means of a circulating water bath. The H+ cells on the other hand, were irradiated on glass in the culture bottles in which they were grown. Again the temperature was maintained at 37°C throughout the irradiations.

Chemical Modifications of Growth Conditions

In many of the experiments presented in this treatise, 5-fluorodeoxyuridine (FUdR)[h] or methotrexate (4-amino-N^{10} methyl pteroylglutamic acid, formerly known as amethopterin)[i] were added to the growth medium to inhibit DNA synthesis. Treatments with both FUdR and methotrexate result in thymidylic acid deficiency. When thymidylic acid is no longer available, DNA synthesis stops. The final concentrations of FUdR used ranged from 2.3×10^{-7} grams per millilitre growth medium (10^{-6} M) to 2.3×10^{-6} grams per millilitre growth medium (10^{-5} M). Methotrexate was used at a concentration of 10^{-8} grams per millilitre growth medium. The DNA synthetic block which is induced by these drugs can be removed by the addition of thymidine. A final concentration of 10 µg thymidine per millilitre growth medium was used for this purpose.

In some experiments it was desirable to build up the number of mitotic figures in the cell population. To this end, colcemid,

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(g) Victoreen Nuclear Instrumentation, Cleveland, Ohio.

(h) Hoffmann-LaRoche Ltd., Montreal, P. Q.

(i) Cyanamid of Canada Ltd., Montreal, P. Q.

which is known to inhibit spindle formation (41), was used. The final concentration employed was 10^{-7} grams colcemid per millilitre growth medium.

To distinguish between cells which were synthesizing DNA and those which were not, various concentrations of H^3TdR[(j)] were tested for incorporation. H^3TdR of specific activity 10 c/m Mole was used at final concentrations of 0.1 µc per millilitre growth medium and 1.0 µc per millilitre growth medium. H^3TdR of specific activity 6.7 c/m Mole was used at a final concentration of 10 µc per millilitre growth medium.

Preparation of Slides

Cell samples were taken at 1, 2, or 4-hour intervals, depending on the type of experiment. The samples were made hypotonic by the addition of 1:8 or 1:4 PBS-water. After fixation in 3:1 ethanol-acetate, the cells were placed on clean microscope slides and stained in 2% aceto-orcein. Mitotic indices usually were determined from counts of 1000 cells. In the experiments in which H^3TdR was used, autoradiographs were made of each of the cell samples after the percentage of mitotic figures had been determined. Slides on which the stained cells were attached, were dipped into Kodak NTB3 nuclear emulsion[(k)] at 45°C, allowed to dry, and then stored in light-tight boxes at 4°C. After an exposure period of from 2½ days to 2 weeks, the slides were developed and the silver grains over the mitotic figures were counted.

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

(k) Ilford Ltd., Essex, England.

Protein and Nucleic Acid Determinations

Protein and nucleic acid determinations were carried out on both control and FUdR-treated cells to study the relative changes induced by the action of FUdR.

All samples from spinner flasks were spun down at 2200 rpm at 0-4°C for 10 minutes. The precipitate was washed twice with PBS and three times with ice-cold 5% trichloroacetic acid (TCA). The nucleic acids were then extracted according to the Schneider procedure (42), using hot TCA as extractant. Extraction was carried out in a thermostatically controlled water-bath with frequent stirring to dispense the precipitate throughout the extractant. DNA and RNA in the extracts were measured colorimetrically in a spectrophotometer-20 by appropriate sugar reactions. DNA was determined by Burton's modification (43) of the diphenylamine reaction (44) and RNA by the Albaum and Umbreit (45) modification of Mejbaum's orcinol estimation (46).

After nucleic acid extraction the tissue residue was washed twice with acetone, ethanol-ether (3:1) and finally ether. The protein in the air-dried residue was then estimated colorimetrically by the method of Lowry et al. (47).

CHAPTER II

RESULTS

Cell Cycle

It was essential for this investigation to know the time relationship between DNA synthesis and mitosis in exponentially multiplying cell cultures. The cell cycle for L-4 cells was established in this laboratory (16) by the methods of Defendi and Manson (48) and Stanners and Till (49). The cells have a mitotic period (M) of 0.5 hours, followed by a G_1 period lasting 5.5-10 hours, a DNA synthesis period (S) of from 6-7.5 hours and finally a G_2 period of 3-3.5 hours preceding the next mitosis. It was found that an increase in generation time was usually due to an increase in the length of the G_1 period. For H.Ep.-2 cells with a generation time of 25 hours, similar studies in this laboratory yielded M, G_1, S and G_2 values of about 0.5, 17, 5 and 3.5 hours respectively.

Mitotic Delay

Mitotic inhibition release curves were obtained by plotting the percentage of cells in mitosis as a function of time following the beginning of irradiation. Linear regression lines were drawn through the rapidly increasing portions of these curves and extrapolated back to zero percent mitoses. The time interval between the beginning of the irradiation and the intersection of the regression line with the time axis was defined as the mitotic delay. A typical mitotic inhibition release curve is shown in Figure 1.

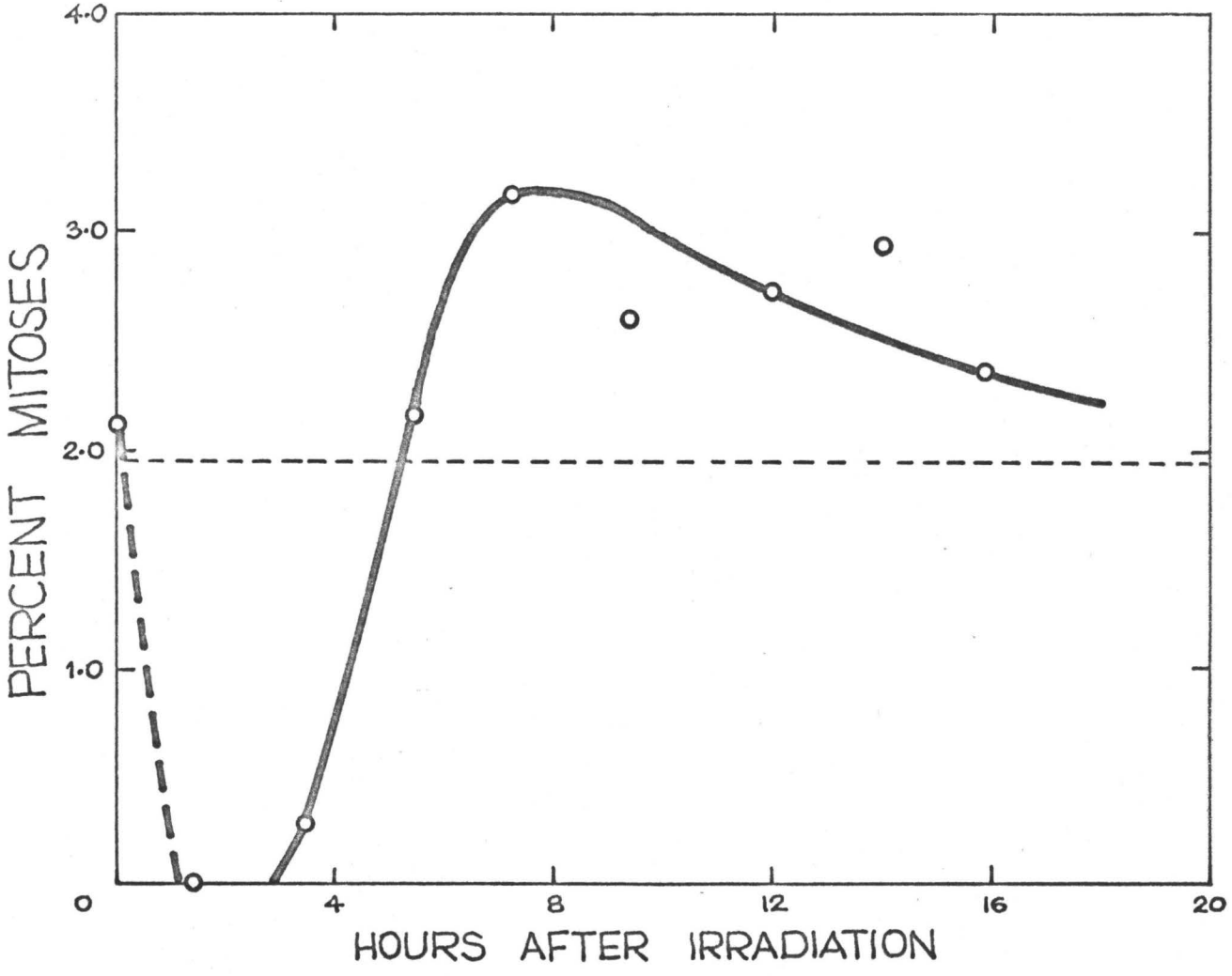

FIGURE 1. RELEASE OF MITOTIC INHIBITION IN MOUSE L-4 CELLS

Previous investigations (8) have indicated that mitotic inhibition involves DNA in some way. One of the ways in which it could be involved is in the repair of radiation damage. Perhaps DNA synthesis is required for such repair. Suppose, for example, repair of radiation damage could not proceed without DNA synthesis. Thus, if synthesis of DNA could be artificially suppressed, then there would be no release of mitotic inhibition until such suppression had been removed. This hypothesis could be tested quite readily if a drug which immediately inhibited all DNA synthesis were available. If such a drug were introduced just prior to an irradiation, and if DNA synthesis were not required for repair of radiation damage, then the cells which were in G_2 at the time of irradiation would enter mitosis after the usual mitotic delay. One would expect the percentage of cells in mitosis to overshoot the normal level and then fall off to zero when G_2 had been depleted of cells. The cells which had been in G_1 and S at the time of irradiation would never reach mitosis since they would be unable to complete the necessary synthesis of DNA. If, however DNA synthesis were required for the repair of radiation damage and if all DNA synthesis were halted through drug action, then not even the G_2 cells could proceed into M. Unfortunately, FUdR and methotrexate, which were chosen to inhibit DNA synthesis to test this hypothesis, do not act immediately upon introduction, but require several hours for full suppression to take effect. This was revealed by a study of the percentage of cells in mitosis in logarithmically growing populations as a function of time after addition of FUdR or methotrexate. Figure 2 represents results obtained with mouse L-4 cells following

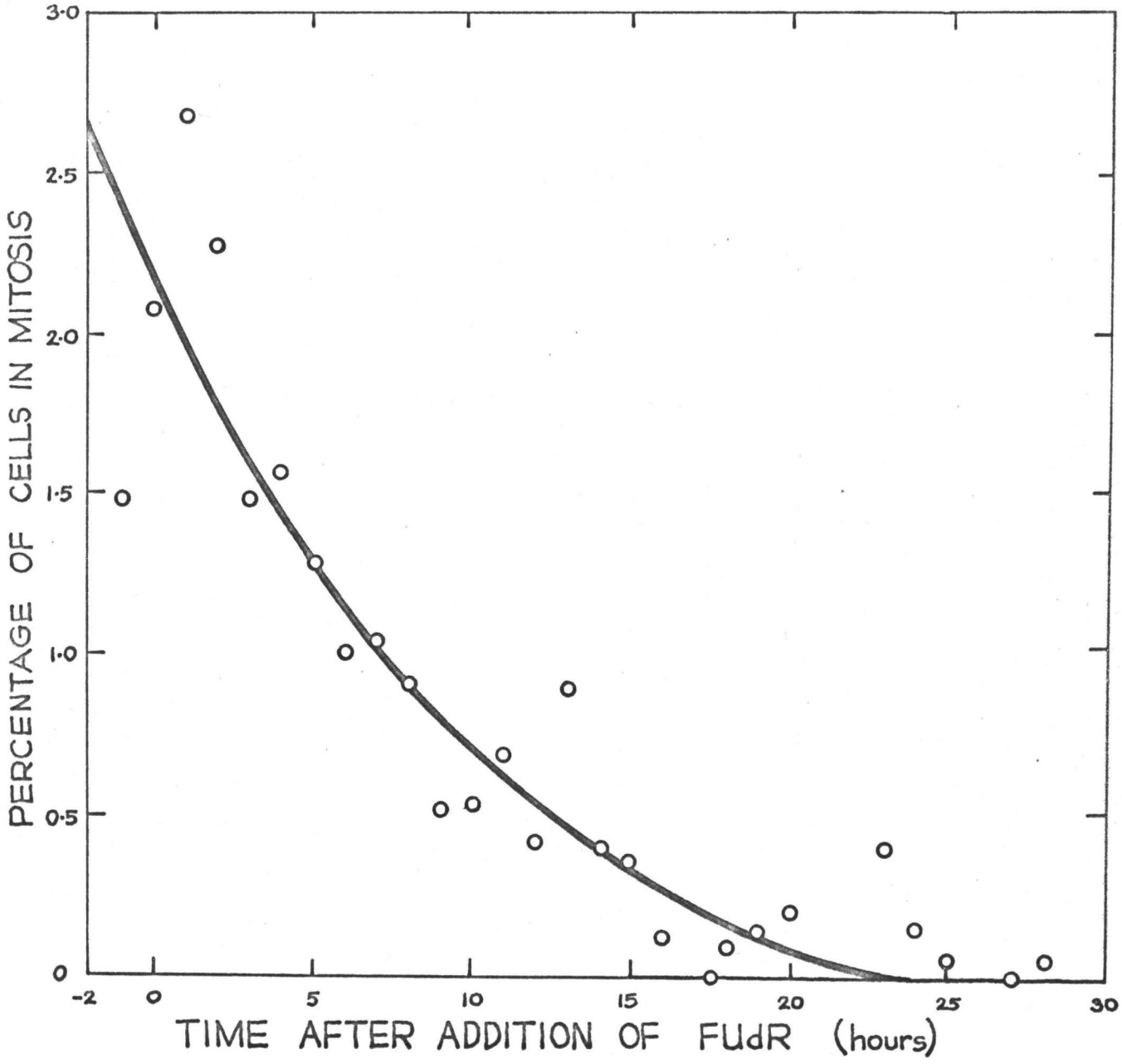

FIGURE 2. RESPONSE OF MOUSE L-4 CELLS TO FUdR (10^{-6}M - 10^{-5}M)

inhibition with FUdR at various concentrations from 10^{-6} M to 10^{-5} M. It was found that there was no significant difference in the action of FUdR at concentrations in this range. At 6 hours after FUdR the percentage of cells in mitosis had dropped to 1.15, at 9 hours to 0.8, at 12 hours to 0.5 and at 23 hours after the addition of FUdR there were essentially no cells in M. The H.Ep.-2 and H+ cells responded in much the same way. Methotrexate appears to act in a similar fashion to FUdR. At 6, 9, 12 and 21 hours after the addition of methotrexate the percentage of L-4 cells in mitosis was respectively, about 1.45, 1.1, 0.75 and 0. During the time required for suppression of DNA synthesis the G_2 phase of the cell division cycle is gradually depleted of cells. As a result the G_2-method outlined above could not be used to determine whether or not DNA synthesis is required for the release of mitotic inhibition. However, it is possible to test the hypothesis in a slightly different manner.

Suppose that prior to irradiation, the cells were treated with FUdR or methotrexate for a time sufficient to inhibit virtually all DNA synthesis. Then regardless of the nature of the mitotic inhibition repair process, no mitoses would be observed after the irradiation, simply because there would be no cell movement out of the S phase. If DNA synthesis were unnecessary for repair of the lesion giving rise to mitotic inhibition, such repair might still occur, even while DNA synthesis were suppressed. If this were the case, and if the suppression then could be removed, mitoses subsequently should appear in the culture after only a short time. For example, this time should be no longer than the duration of G_2 if DNA synthesis had been suppressed

long enough for the radiation damage to be repaired. On the other hand, if DNA synthesis were necessary for repair of the mitotic inhibition lesion, then such repair could not occur until the synthesis were reinitiated. As a result, after reversal of the action of the suppressing agent, mitoses would not be expected to appear in the culture for a period of time which, depending upon how much repair could occur without DNA synthesis, might even be as large as the usual mitotic delay.

DNA synthesis can, in fact, be reinitiated after FUdR or methotrexate treatment simply by the addition of TdR. TdR reversal of FUdR inhibition in L-4 cells is shown in Figure 3. In the various experiments performed, TdR at a concentration of 10 μg/ml was added at 12, 14, 16, 18 or 21 hours after the initiation of FUdR treatment and seemed to reverse the suppression caused by 2×10^{-6} M, 5×10^{-6} M or 10^{-5} M FUdR equally well, regardless of the time at which it was added. Mitoses started appearing at 3-3½ hours after the addition of TdR. Since this is just the duration of G_2, this indicates that DNA synthesis must begin immediately upon addition of TdR. The rapid rise at 6-8 hours represents the cells which had been unable to enter S and so had piled up at the end of G_1. This is earlier than would be expected normally, and indicates an abbreviated S period such as that mentioned by Till for L cells (35) and Priest for human diploid cells (50). Results for TdR reversal of the action of methotrexate were found to show much the same pattern.

Thus, it is possible, through the use of FUdR or methotrexate and TdR, to test the hypothesis that DNA synthesis is required for

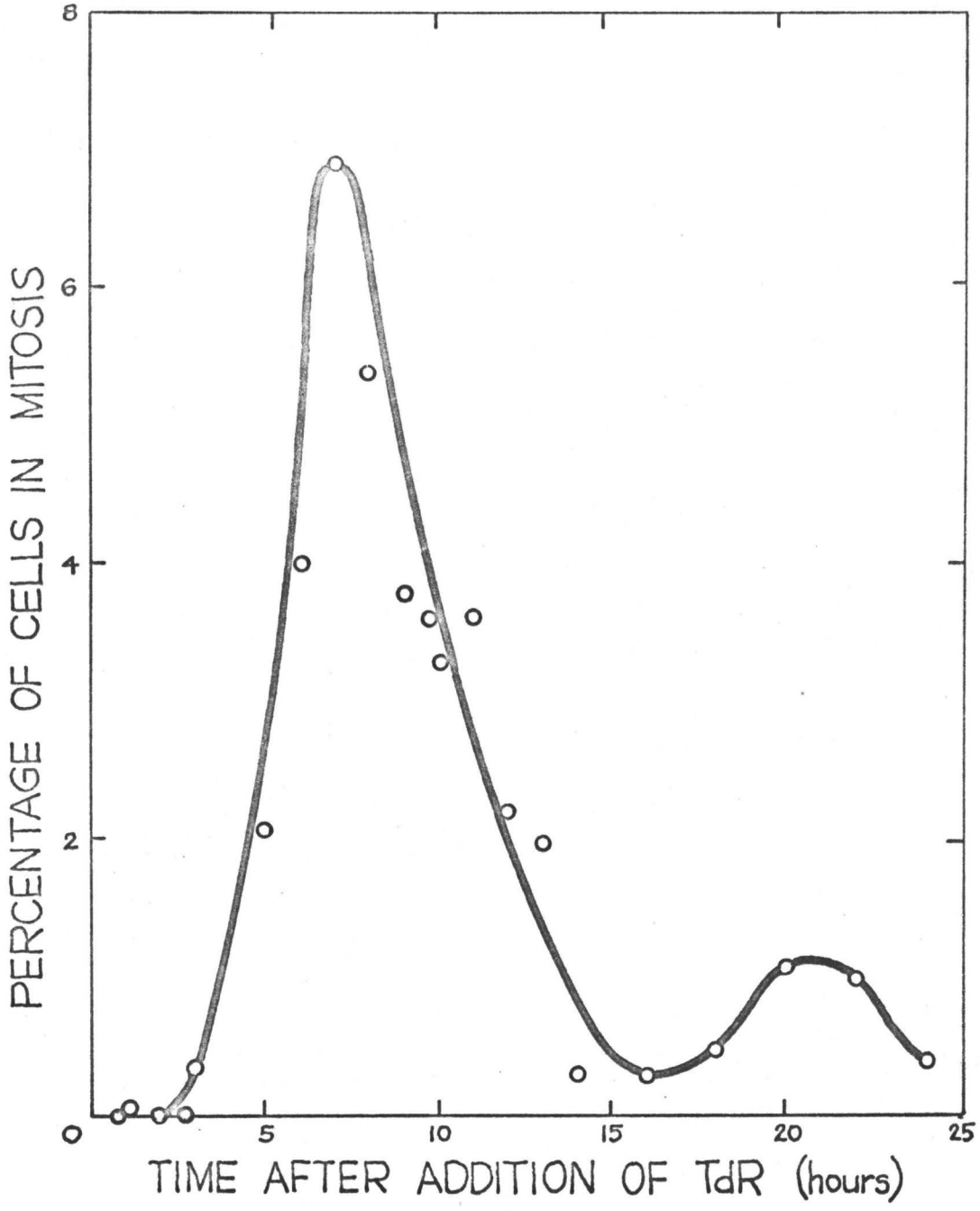

FIGURE 3. RELEASE OF FUdR-BLOCK IN MOUSE L-4 CELLS BY ADDITION OF TdR (10 μg/ml)

the release of mitotic inhibition. Treatment with FUdR or methotrexate for 12 or more hours prior to irradiation will virtually inhibit all DNA synthesis. If TdR were added at the time of irradiation one would expect mitoses to start appearing after the usual mitotic delay, regardless of whether or not DNA synthesis were required for repair of radiation damage. However if TdR were added 4, 8 or 12 hours after the irradiation and if DNA synthesis were required for the release of mitotic inhibition, the mitotic delay could be increased by as much as 4, 8 or 12 hours, respectively. If DNA synthesis were not required for this release, one would expect either the usual mitotic delay or a delay equal to the sum of the G_2 phase plus the duration of the interval between irradiation and the addition of TdR, which ever period was the greater.

Figure 4a is representative of the mitotic inhibition release curves obtained when TdR was added to FUdR-treated cell populations at various times after an irradiation. Here the dose was 600 rads and had been preceded by a 12 hour FUdR treatment. Figure 4b contains the same data as Figure 4a except that instead of smooth inhibition release curves, linear regression lines have been drawn for the rapidly rising region in each case. Standard errors for the linear regression lines are indicated under the time scale. A dose of 600 rads normally causes a mitotic delay of 6-10 hours in mammalian cell populations which have not been pre-treated with FUdR. A similar delay was observed in FUdR-treated cultures to which TdR was added at the time of irradiation. In the experiment depicted in Figure 4, the culture which received TdR at the time of irradiation underwent a mitotic

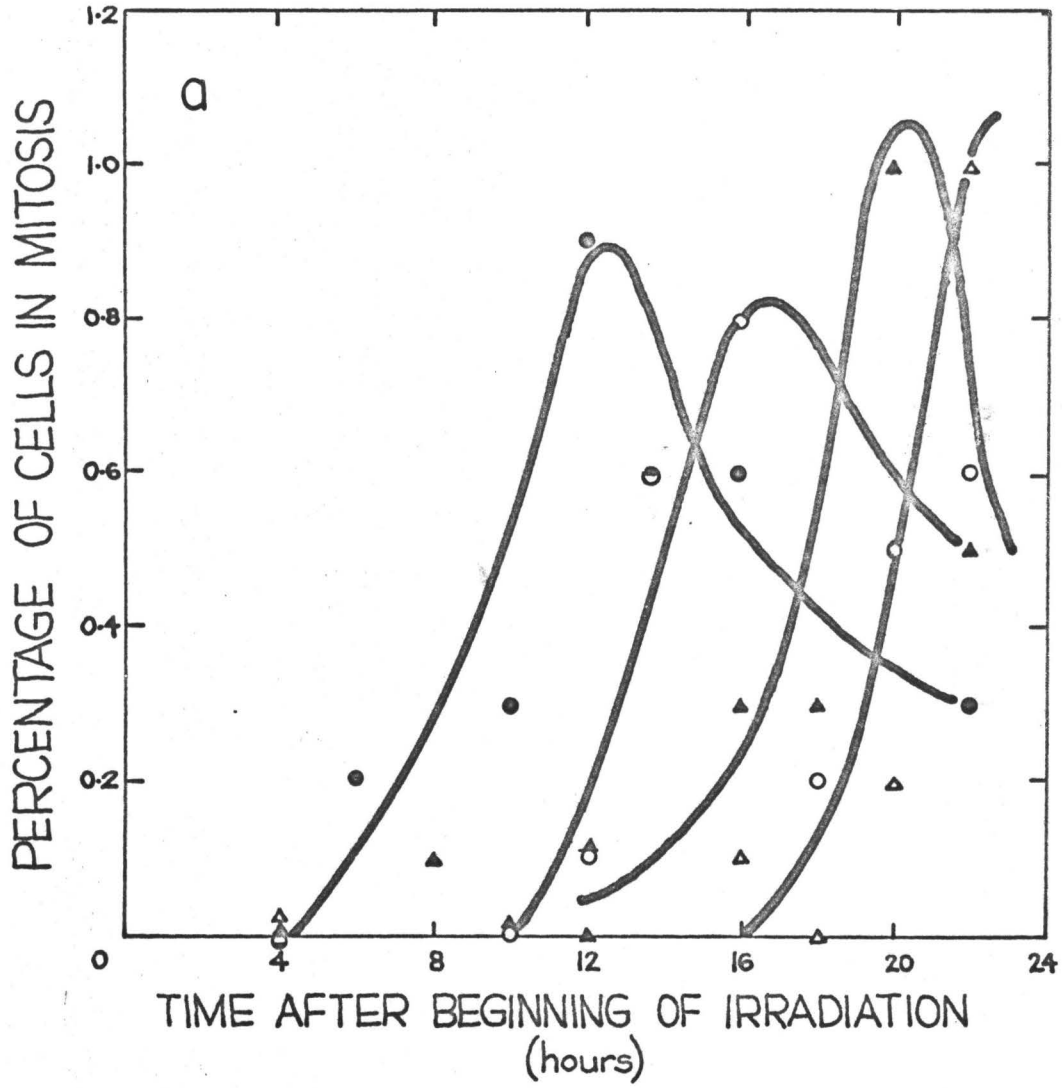

FIGURE 4 RELEASE OF RADIATION-INDUCED MITOTIC INHIBITION IN MOUSE L-4 CELLS. THE CELLS RECEIVED 600 RADS IRRADIATION 12 HOURS AFTER TREATMENT WITH 2×10^{-6}M FUdR. THE FUdR BLOCK SUBSEQUENTLY WAS RELEASED BY ADDITION OF TdR (10 µg/ml) AFTER A FURTHER 0 HOURS (●); 4 HOURS (○); 8 HOURS (▲); 12 HOURS (△).

(a) MITOTIC INHIBITION RELEASE CURVES.

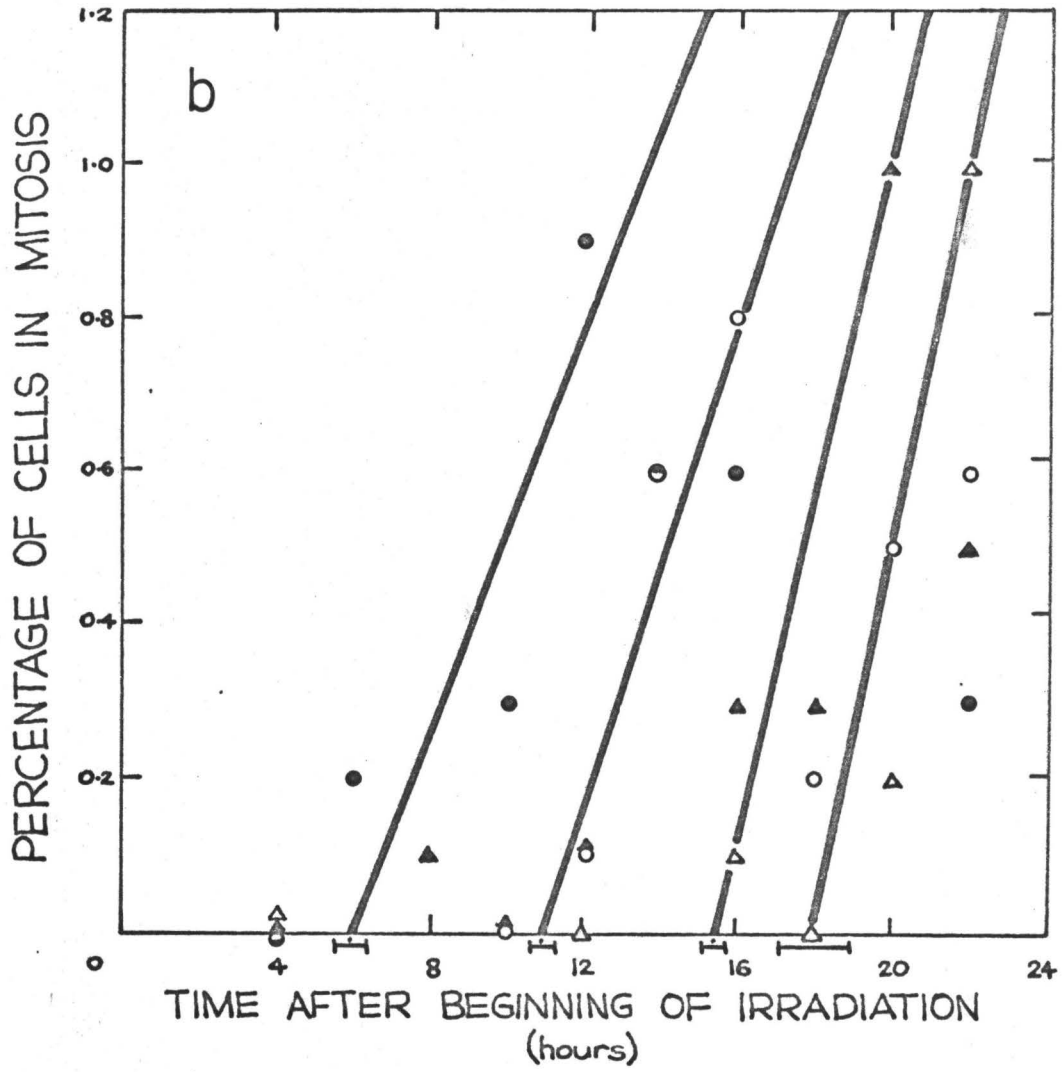

FIGURE 4 RELEASE OF RADIATION-INDUCED MITOTIC INHIBITION IN MOUSE L-4 CELLS. THE CELLS RECEIVED 600 RADS IRRADIATION 12 HOURS AFTER TREATMENT WITH 2×10^{-6}M FUdR. THE FUdR BLOCK SUBSEQUENTLY WAS RELEASED BY ADDITION OF TdR (10 µg/ml) AFTER A FURTHER 0 HOURS (●); 4 HOURS (○); 8 HOURS (▲); 12 HOURS (△).

(b) LINEAR REGRESSION LINES AS DETERMINED FROM THE EXPERIMENTAL DATA IN THE FAST-RISING PORTIONS OF THE MITOTIC INHIBITION RELEASE CURVES.

delay of 6.1 hours. If DNA synthesis were required for the release of mitotic inhibition and if TdR were added at 4, 8 or 12 hours after the irradiation, then the delay could be increased by as much as 4, 8 or 12 hours respectively. In this case, one would expect mitotic delays as great as 10.1, 14.1 and 18.1 hours. However, if DNA synthesis were not required for the release of mitotic inhibition one would expect delays no greater than 7.5, 11.5 and 15.5 hours in cultures to which TdR was added at 4, 8 and 12 hours after the irradiation. The average mitotic delays observed for such cultures were respectively 11.0, 15.4 and 18.0 hours, i.e. 4.9, 9.3 and 11.9 hours longer than the mitotic delay suffered by the control. Similar results were obtained with all cell lines tested. A summary of these results is presented in table I.

TABLE I

MITOTIC INHIBITION RELEASE (Hours after control)

Time of addition of TdR (hours after irradiation)	4			8			12		
Expected times of release if repair cannot proceed without DNA synthesis	4			8			12		
Cell Line	L-4	H.Ep.-2	H+	L-4	H.Ep.-2	H+	L-4	H.Ep.-2	H+
Expected times of release if repair can proceed without DNA synthesis	0	0	0	2.9	0.9	3.3	6.9	4.9	7.3
Observed times of release (averaged)	4.2	3.9	4.8	8.4	4.6		11.9	6.8	12.8

Although control cultures in the three cell lines used underwent different mitotic delays, the delays all fell within the same range. The expected times of release presented in the table were calculated separately for each cell line because of the slight differences observed in the mitotic delays of the controls.

It would appear from table I that repair of the mitotic inhibition lesion cannot proceed without DNA synthesis. However, since the pre-irradiation treatment with FUdR lasted at least 12 hours in the experiments presented so far, one may wonder what has happened to cellular processes other than DNA synthesis during that time. Perhaps one could imagine RNA and protein synthesis also being inhibited during the protracted treatment with FUdR. If, in fact, they, as well as DNA synthesis were inhibited, one could no longer be sure that it was only DNA synthesis which was required for the release of mitotic inhibition. Thus it was essential to investigate the action of FUdR on cellular RNA and protein synthesis as well as on DNA synthesis.

The colorimetric determinations of DNA, RNA and protein content in L-4 cells treated with FUdR are represented in Figures 5, 6 and 7 respectively. Each of the DNA and RNA values represents the average of two samples and each protein value is the average of four samples. One can see that FUdR starts inhibiting DNA synthesis almost immediately, whereas there does not seem to be inhibition of RNA and protein synthesis until much later. RNA and protein content in the FUdR treated cultures do not deviate significantly from the control values until about 14 and 18 hours respectively. Preliminary

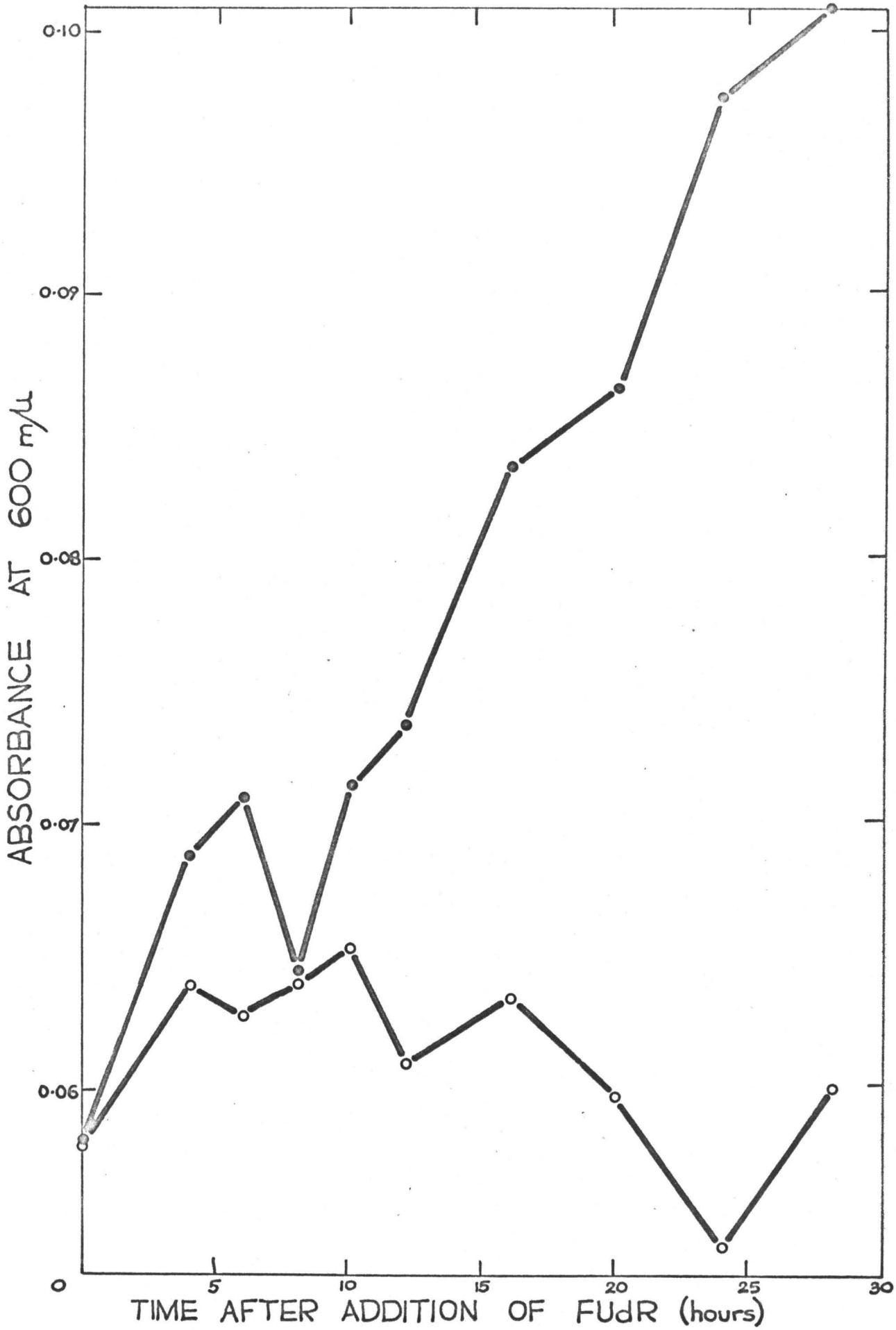

FIGURE 5. EFFECT OF FUdR (2×10^{-6} M) ON DNA IN MOUSE L-4 CELL CULTURES AS DETERMINED BY THE DIPHENYLAMINE REACTION.
● — CONTROL ○ — FUdR TREATED

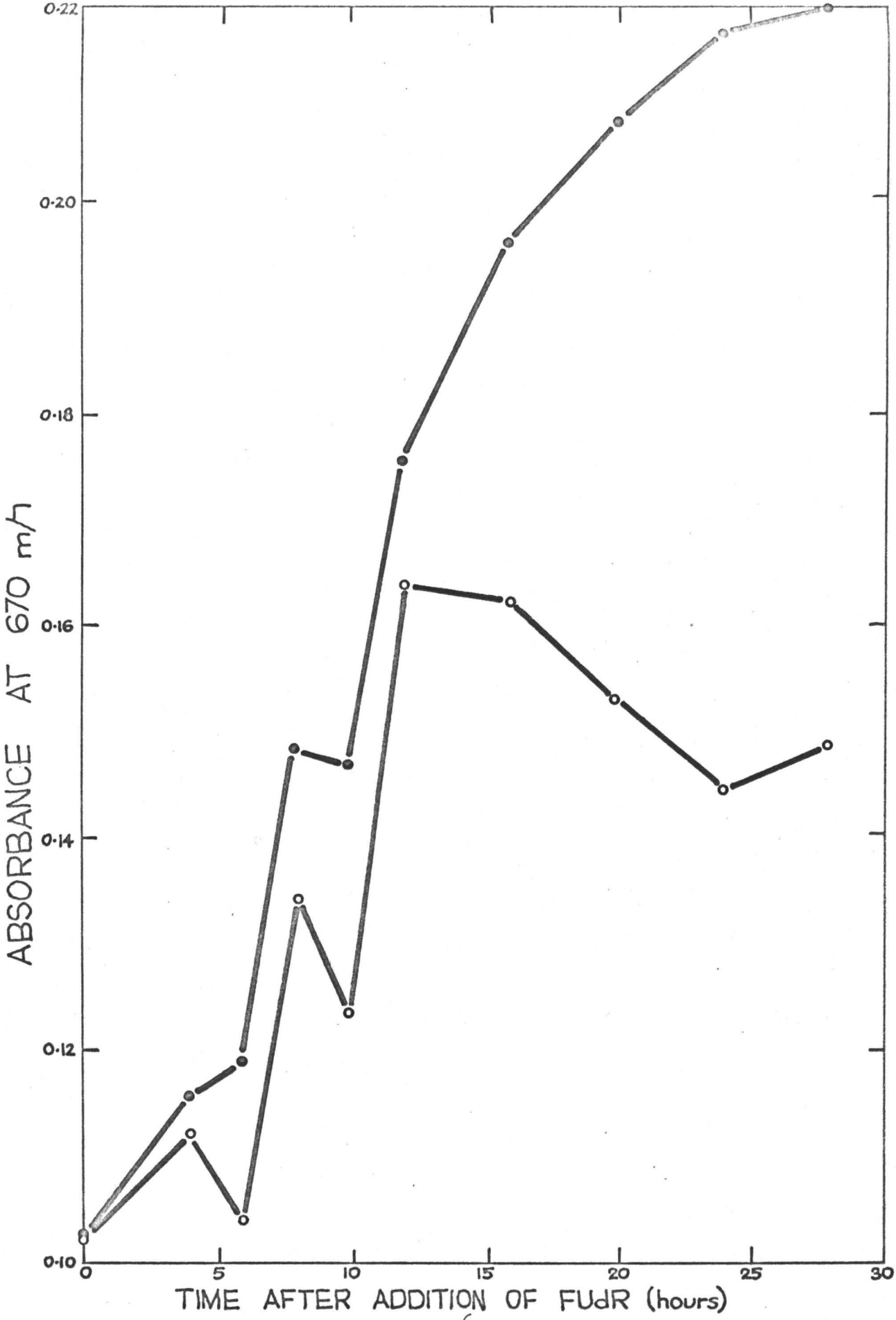

FIGURE 6. EFFECT OF FUdR (2×10^{-6}M) ON RNA IN MOUSE L-4 CELL CULTURES AS DETERMINED BY THE ORCINOL REACTION
●--- CONTROL ○---FUdR TREATED

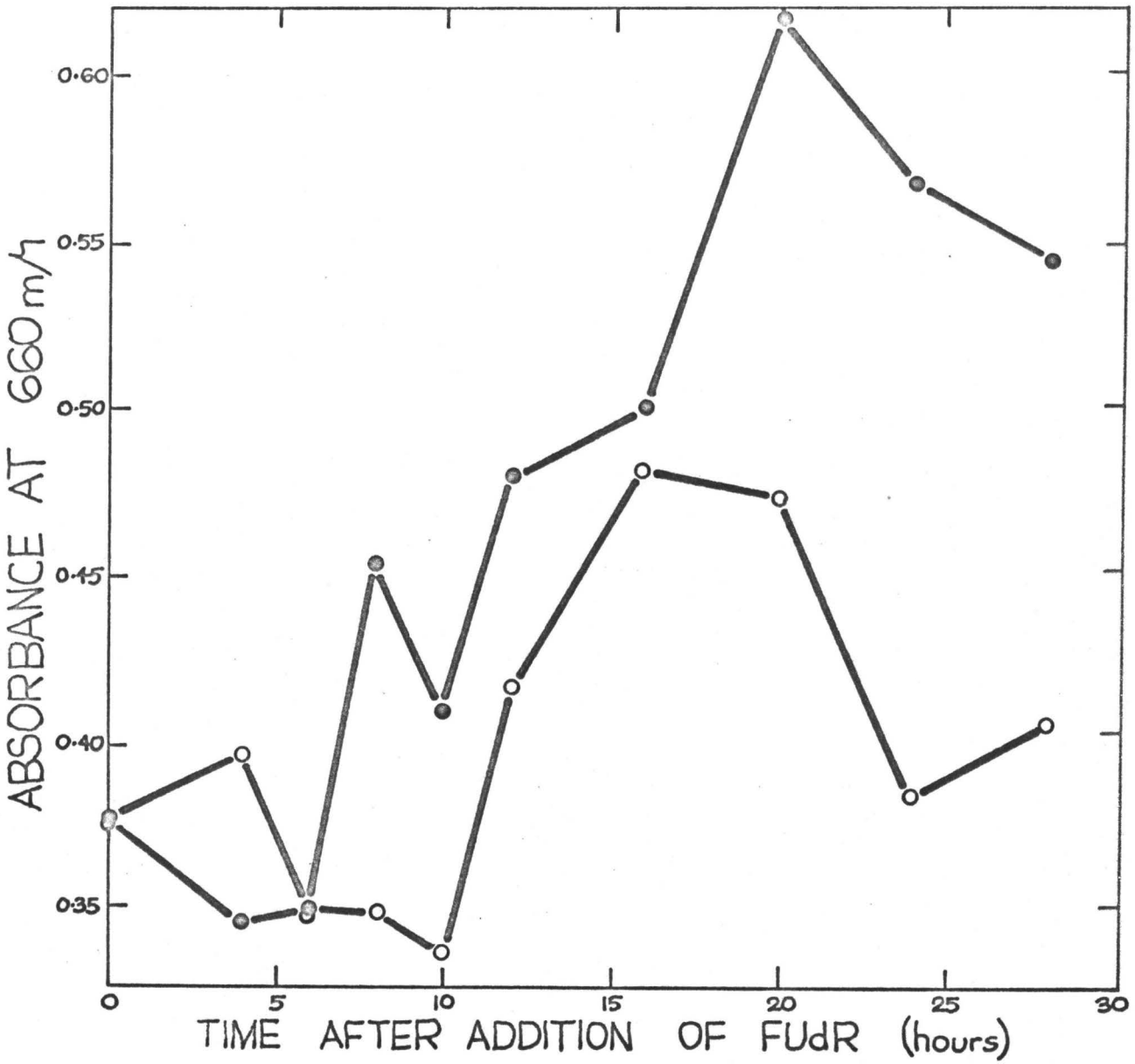

FIGURE 7. EFFECT OF FUdR (2×10^{-6}M) ON PROTEIN IN MOUSE L-4 CELL CULTURES AS DETERMINED BY THE METHOD OF LOWRY ET AL (47)
●--- CONTROL ○---FUdR TREATED

experiments with H.Ep.-2 cells indicate that RNA and protein synthesis are not inhibited until about 24 hours after the introduction of FUdR.

It seems then that in the experiments in which the irradiation of L-4 cells was carried out at 16 hours after the initiation of FUdR treatments, one can not be sure that it is only DNA synthesis which is required for the release of mitotic inhibition, since there may be inhibition of RNA synthesis by this time. However, if one observed similar mitotic delays in experiments in which the irradiation was conducted on the one hand at a time when there was inhibition of DNA synthesis but not of RNA or protein synthesis, and on the other hand, at times at which there could be inhibition of all three, one could say with some certainty that it was DNA synthesis which was required for the release of mitotic inhibition. Results from an experiment in which L-4 cells were irradiated at 9 hours after the initiation of FUdR treatment are shown in Figure 8. At 9 hours after the addition of FUdR there is about a 35% inhibition of DNA synthesis, but evidently no inhibition of RNA or protein synthesis. The mitotic delays observed in cultures to which TdR was added at 0, 4, 8 and 12 hours after irradiation, were respectively 6.6, 11.0, 14.8 and 18.6 hours. Hence the delay suffered by the control was increased by 4.4, 8.2 and 12.0 hours when TdR was added at 4, 8 and 12 hours after irradiation. These values are very close to those expected if DNA synthesis is required for repair of the mitotic inhibition lesion (see table I). Likewise they are similar to the values obtained in the experiments in which the mouse cells were irradiated at from 12-16 hours after the initiation of FUdR treatment.

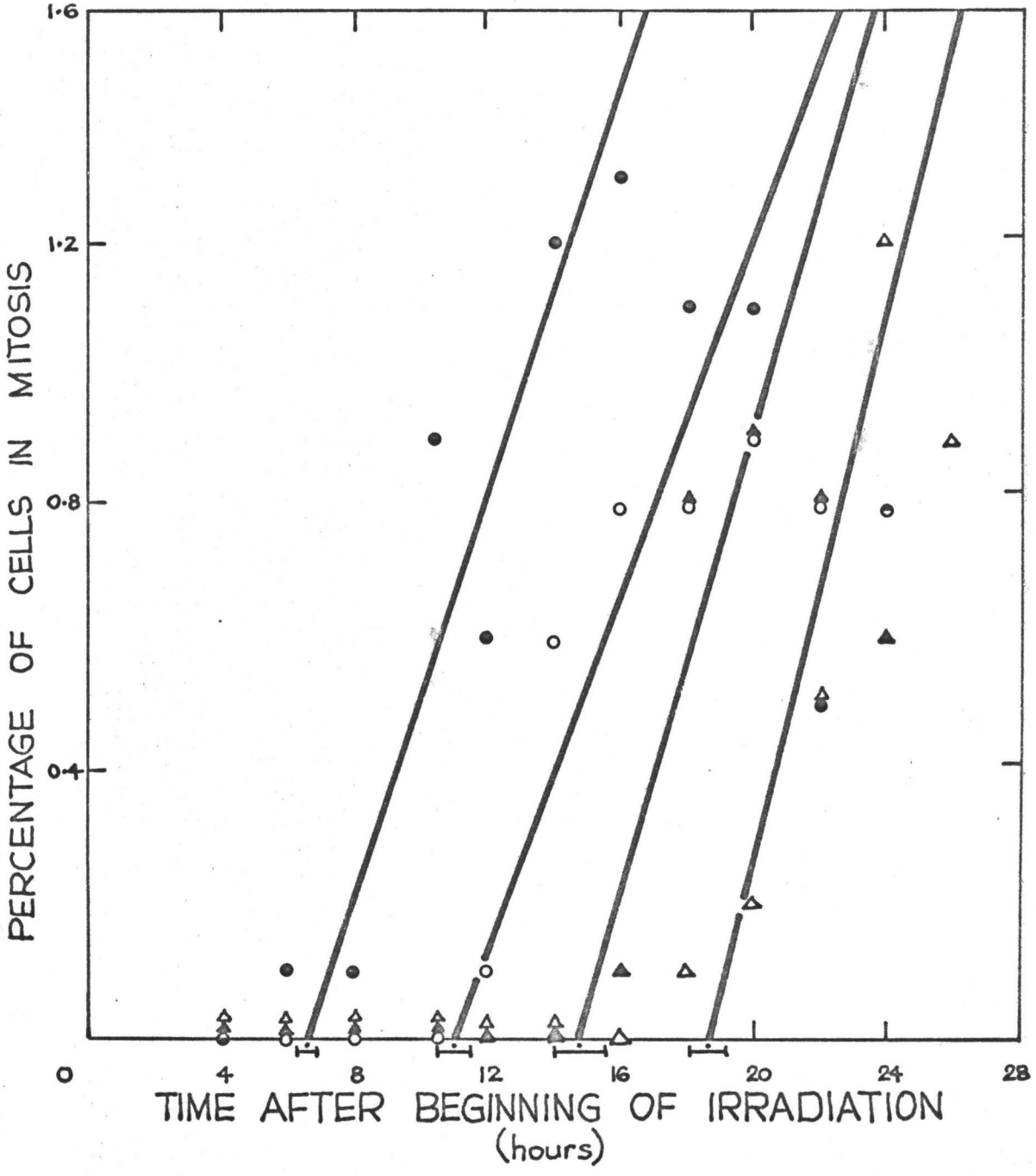

FIGURE 8. RELEASE OF RADIATION-INDUCED MITOTIC INHIBITION IN MOUSE L-4 CELLS. THE CELLS RECEIVED 600 RADS IRRADIATION 9 HOURS AFTER TREATMENT WITH 2 x 10^{-6}M FUdR. THE FUdR BLOCK SUBSEQUENTLY WAS RELEASED BY ADDITION OF TdR (10 µg/ml) AFTER A FURTHER 0 HOURS (●); 4 HOURS (○); 8 HOURS (▲); 12 HOURS (△)

It appears, then, that mouse L cells which have had a 9 hour pre-irradiation treatment with FUdR can not be relieved of the mitotic inhibition until the FUdR blockage of DNA synthesis is removed. Much less emphatic data were obtained when the irradiation was proceeded by a 6 hour FUdR treatment. Results from such an experiment are represented in Figure 9. Here, for TdR added at 4, 8 and 12 hours after irradiation, the mitotic delay suffered by the control was increased by 1.9, 3.4 and 4.6 hours respectively, which is much less than would be expected if repair had been halted by the cessation of DNA synthesis. Three of the four mitotic inhibition release curves obtained in this experiment were not characterized by the usual rapidly increasing region followed by a gradual leveling off, but rather by a rapidly increasing region followed by a decrease and then a further increase in the percentage of cells in mitosis. Thus, when regression lines were drawn through the first rapidly increasing region of these curves, the mitotic delays so determined for cultures other than the control were much shorter than observed in previous experiments. It is very probable that a 6 hour pre-irradiation treatment with FUdR is not sufficient to inhibit DNA synthesis to such an extent that no repair of the mitotic inhibition lesion can take place. However, there was not, in any of the cultures, a release of mitotic inhibition before the addition of thymidine.

If, as is indicated by the FUdR experiments, DNA synthesis is required for the release of mitotic inhibition one could expect to see unscheduled synthesis of DNA in cells which have been irradiated. For example, if an asynchronous population were subjected to a radiation

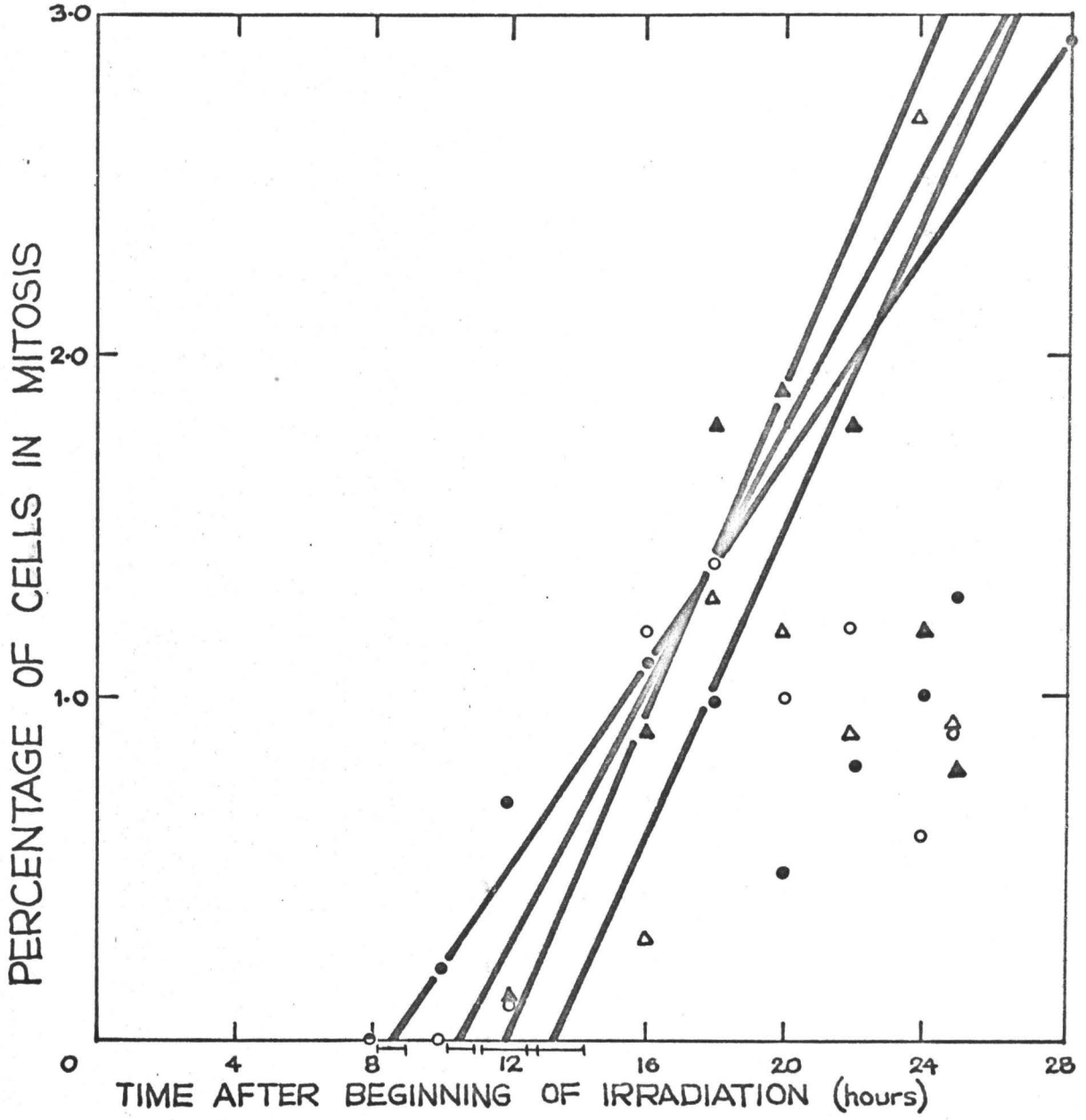

FIGURE 9. RELEASE OF RADIATION-INDUCED MITOTIC INHIBITION IN MOUSE L-4 CELLS. THE CELLS RECEIVED 600 RADS IRRADIATION 12 HOURS AFTER TREATMENT WITH 2 x 10^{-6}M FUdR. THE FUdR BLOCK SUBSEQUENTLY WAS RELEASED BY ADDITION OF TdR (10 μg/ml) AFTER A FURTHER 0 HOURS (●); 4 HOURS (○); 8 HOURS (▲); 12 HOURS (△)

dose which would yield a mitotic delay shorter than the length of G_2, then the cells seen in mitosis during the first 3-3½ hours after the irradiation would have been in G_2 at the time of the irradiation. Thus, if our hypothesis were correct, introduction of H^3TdR at approximately the same time as irradiation of the culture should result in incorporation of tritiated thymidine into the inhibited G_2 cells. Such incorporation should be able to be observed autoradiographically in these cells after the inhibition had been released and the cells had entered mitosis.

In the first experiments conducted, H^3TdR (10 c/mM) was added at concentrations of 0.1 and 1.0 μc per ml growth medium at the same time as L-4 cells were irradiated with from 50-150 R. However, no labeling above background was observed in G_2 cells. More positive results were obtained with a higher concentration of H^3TdR. Results from an experiment in which H^3TdR (6.7 c/mM, 10 μc/ml) was added two minutes prior to an irradiation of 117 R are presented in table II and Figure 10.

The background per cell area was determined from averages of grain counts taken in three or four average cell areas in the vicinity of the metaphase cells. The limits shown are the 95% confidence limits. For some unknown reason a consistently high background was observed in the sample taken five minutes after the irradiation. At this time none of the cells observed in mitosis had labeling above background, however roughly 33% of the non-metaphase cells had more than 25 grains/nucleus so the label presumably was being incorporated. At 1, 2 and 3 hours after the irradiation about 30% of the metaphase

figures observed had more grains than the corresponding background. At 5½ hours after the irradiation about 83% of the cells in mitosis were labelled. This is what one would expect, since at 5½ hours the cells which are being observed were in S at the time of addition of H^3TdR.

TABLE II

INCORPORATION OF H^3TdR INTO IRRADIATED MOUSE L-4 CELLS

Time after irradiation that sample was fixed	Number of grains/cell area in background ± 1.96 S.E.	Number of grains/metaphase greater than background /cell area	Number of metaphase cells counted											
			0	0-1	1-2	2-3	3-4	4-5	5-6	6-7	7-10	10-20	20-30	30-60
5 minutes	3.6 ± 1.08		34											
1 hour	0.5 ± 0		36		6	6		1	1					
2 hours	0.4 ± 0.02		36	2	2	9		2		1				
3 hours	0.8 ± 0.01		38		4	3	4	3	1		1		1	
5½ hours	0 ± 0		5			3	2	2	3	3	5	2	2	3

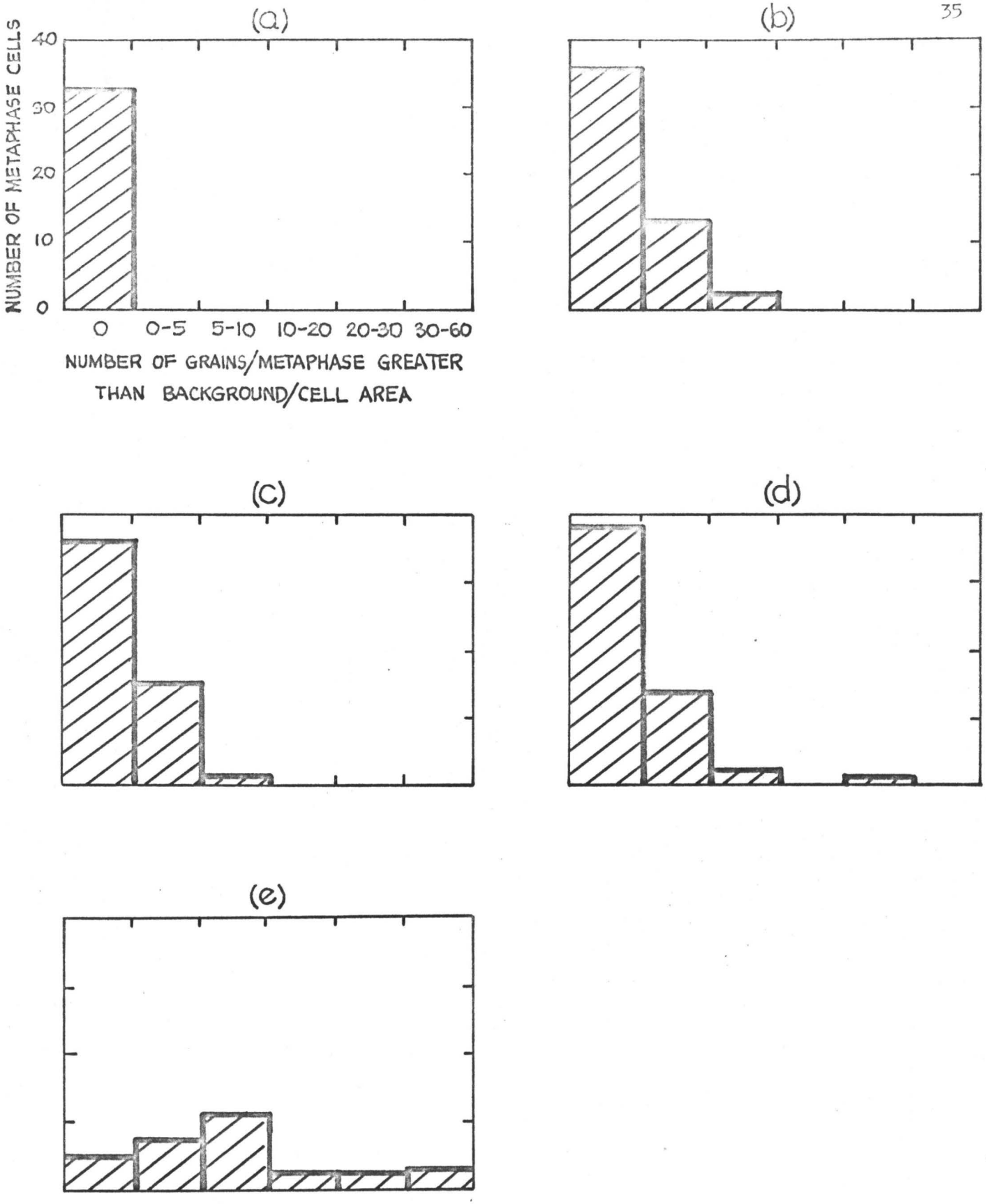

FIGURE 10. INCORPORATION OF H_3TdR (6.7 c/mM, 10 μc/ml) INTO IRRADIATED (117 RADS) MOUSE L^{-}_{4} CELLS. (a) 5 MINUTES AFTER IRRADIATION; (b) 1 HOUR AFTER IRRADIATION; (c) 2 HOURS AFTER IRRADIATION; (d) 3 HOURS AFTER IRRADIATION; (e) 5½ HOURS AFTER IRRADIATION

CHAPTER III

DISCUSSION

In this investigation γ-radiation-induced mitotic inhibition was studied in three types of mammalian cells, all of which were grown *in vitro*. Of particular concern was the involvement of cell DNA in the release of mitotic inhibition. It was important, therefore, to know the time during the cell cycle when DNA is synthesized and the time relationship between the period of DNA synthesis and the period of mitosis.

The mammalain cell cycle has been divided into four stages (15): a mitotic period (M) during which cell division takes place, followed by G_1, a pre-DNA synthesis period, S, a period during which DNA is synthesized and finally G_2, a pre-mitotic, post-DNA synthesis period.

The cell cycle of mouse L-4 cells, on which most of the studies were conducted, was established in this laboratory (16) by the use of two procedures. One method involved pulse labeling of the cells which were synthesizing DNA (48) and the other involved continuous labeling of the cell population (49). In both cases grain counts over metaphases were plotted as a function of time, and the cell cycle parameters were derived from the curves. The lengths of G_2 and S were found to be 3-3.5 hours and 6-7.5 hours, respectively. The duration of M, determined by the use of an equation developed by Stanners and Till (50) was 0.5 hours. The duration of G_1, found by subtraction of G_2 + S + M from the generation time of the population, was 5.5-10 hours. Use of the

continuous labeling technique on human H.Ep.-2 cells with a doubling time of 25 hours, yielded G_2, S, M and G_1 periods of about 3.5, 5, 0.5 and 17 hours respectively. The cell cycle was not determined for the hamster H+ cells.

Although several workers (4, 51, 12) have reported an increase in sensitivity to mitotic delay as mammalian cells pass from G_1 to S to G_2, investigations in this laboratory (16) indicate no significant variation in sensitivity around the cell cycle.

The phenomenon of mitotic inhibition, which has been described as the immediate, temporary disappearance of mitoses following irradiation of a cell population, is claimed to be due primarily to a radiation-induced block located in late G_2 (18, 52). The release of mitotic inhibition is characterized by a rapid increase in the percentage of cells in mitosis at a time which is dependent on the magnitude of the dose. The time duration between the irradiation and the reappearance of mitoses in the cell population is called the mitotic delay. The mitotic delay may be determined in a number of ways, all of which involve plotting the percentage of cells in mitosis as a function of time. One way which has been used recently (32) considered the mitotic delay as the time duration between irradiation and the point at which the percentage of cells in mitosis is 25% of the average value in an unirradiated population. Another method involves drawing the best smooth curve to zero percent mitoses. In this case the time interval between the beginning of the irradiation and the intersection of the curve with the time axis is defined as the mitotic delay. In this investigation, linear regression lines were drawn through the rapidly increasing

portions of the mitotic inhibition release curves and extrapolated back to zero percent mitoses. The time interval between the beginning of the irradiation and the intersection of the regression line with the time axis was defined as the mitotic delay. Such regression lines greatly facilitate the calculation of standard errors for the mitotic delays observed.

Involvement of cell DNA in the mitotic inhibition response has been indicated by experiments in which treatment with bromodeoxyuridine (BUdR) increased the sensitivity of L cells to mitotic inhibition (32). BUdR is an analogue of deoxythymidylic acid (TdR) and is incorporated specifically into the DNA of mammalian cells. The experiments with 5-fluorodeoxyuridine (FUdR) and methotrexate (4-amino-N^{10}-methyl pteroylglutamic acid) which are presented in this treatise show that DNA synthesis is required for the release of radiation-induced mitotic inhibition. Treatments with both FUdR and methotrexate result in thymidylic acid deficiency. FUdR is a strong and specific inhibitor of the enzyme thymidylate synthetase, (26) which catalyzes the methylation of deoxyuridylic acid to yield thymydilic acid. Methotrexate acts by inhibiting the enzymes folic acid reductase and dihydrofolic acid (THFA) from folic acid. Derivatives of THFA act as coenzymes in the thymidylate synthetase catalyzed formation of thymidylic acid (53). Thus treatment with either FUdR or methotrexate results in an inhibition of DNA synthesis via an induced thymidylic acid deficiency.

The effect of FUdR on the percentage of mitoses in logarithmically growing mouse L cells is represented in Figure 2. It was found that there was no significant difference in the action of FUdR at concentrations in the range from 10^{-6} to 10^{-5} M. It is obvious from the figure that it took several hours for complete suppression of DNA synthesis to take effect. The percentage of cells in mitosis did not fall to zero until approximately 23 hours after the addition of FUdR. Hsu et al. (54) observed that it took approximately 10 hours for the mitotic index of mouse L-M cells to drop to zero after the addition of 0.01 µg/ml FUdR. Such a rapid reduction of the percentage of cells in mitosis was rarely observed in this laboratory. A similar response was observed in H.Ep.-2 and H+ cells treated with FUdR at concentrations of 2×10^{-6} M and 10^{-5} M respectively. There is a tremendous discrepancy between these results and those of Eidinoff and Rich (55) who claimed that three hours after the addition of FUdR at a concentration of 4×10^{-7} M, the metaphase frequency of H.Ep.-1 cells dropped from the control value of 2% to zero. Both H.Ep.-1 and H.Ep.-2 are human epithelial cell lines but H.Ep.-1 was isolated from a cervical carcinoma, whereas H.Ep.-2 was isolated from a carcinoma of the larynx. It is unlikely that differences in doubling time (and hence in the length of G_1) alone could account for the differences in the response to FUdR. It is possible that a small amount of thymidine is being supplied by the fetal calf serum with which the medium was supplemented in this laboratory. As has been mentioned earlier, attempts were made to remove any thymidine from the fetal calf serum by dialysis in doubly distilled water and in phosphate buffered saline. Unfortunately, dialyzed serum

supported growth only for short periods of time and consequently most of the experiments were carried out with cells growing in undialyzed serum.

Methotrexate at a concentration of 10^{-8} g/ml appears to act in much the same fashion as 10^{-6} to 10^{-5} M FUdR. The percentage of mouse L cells in mitosis dropped to zero at approximately 21 hours after the addition of methotrexate at such a concentration.

The inhibition of mitotic activity caused by the treatment of mammalian cells with FUdR or methotrexate can be reversed by the addition of thymidine (TdR) or analogues of thymidine (e.g., 54, 55). TdR reversal of the action of FUdR on mouse L cells is shown in Figure 3. TdR added at a concentration of 10 μg/ml at 12, 14, 16, 18 or 21 hours after the initiation of treatment with FUdR at 2×10^{-6} M, 5×10^{-6} M or 10^{-5} M appeared to reverse the suppression of mitotic activity. Mitoses started appearing at 3-3½ hours after the addition of TdR. Since this is just the duration of G_2, some DNA synthesis must begin immediately upon addition of TdR. This is in accord with the hypothesis put forward by Till and Whitmore that FUdR blocks the passage of cells through the S period but not their passage through other periods of the cycle. As a result, after several hours of FUdR treatment, G_2, M and G_1 will be depleted of cells and there will be a large accumulation of cells at the beginning of S, and some cells which were unable to complete DNA synthesis will be distributed throughout S. It is likely the cells near the end of S which are observed at 3-3½ hours after addition of TdR. Supposedly these cells would have to undergo very little DNA synthesis before completing the

S phase and consequently, after the addition of TdR, would reach M after a time approximately equal to the length of G_2. Hsu *et al.* (54) also observed a release of mitotic inhibition at 3 to 4 hours after the addition of TdR to FUdR-treated mouse LM cells. A rapid rise in the percentage of cells in mitosis was observed at 6-8 hours following the initiation of TdR reversal of the FUdR block. This represents the cells which had been unable to enter S and so had piled up at the end of G_1. This is about 3 hours earlier than would be expected normally, and indicates that the duration of the S period had been reduced during the treatment with FUdR. Such an abbreviated S also was observed by Till for mouse L cells (35) and Priest for human diploid cells (50). A similar pattern was observed for TdR reversal of the action of methotrexate on mouse L cells.

Since the inhibition of DNA synthesis resulting from treatment with FUdR or methotrexate can be reversed by the addition of TdR, and since these effects are manifested in the mitotic activity of cells, one can readily use such a system to test the hypothesis that DNA synthesis is required for the release of radiation-induced mitotic inhibition. Thus, cells could be treated with FUdR or methotrexate for a time sufficient to inhibit virtually all DNA synthesis and reduce the mitotic index to zero. Subsequent irradiation, therefore, would give rise to the mitotic inhibition lesion, but the repair of the lesion could not be observed. However, would this repair actually occur? Certainly, if DNA synthesis were unecessary for the repair, it should, at least to some extent. On the other hand, if DNA synthesis were required for repair of the lesion, no such repair could take place

until the suppression were removed. Consequently, after reversal of the action of the inhibitory drug, the time of appearance of mitoses in the culture would depend greatly upon how much repair could occur without DNA synthesis. Thus, for example if TdR were added 4, 8 or 12 hours after the irradiation and if DNA synthesis were required for the release of mitotic inhibition, the mitotic delay could be increased by as much as 4, 8 or 12 hours respectively. Of course, if TdR were added at the time of irradiation, one would expect mitoses to start appearing after the usual mitotic delay, regardless of whether or not DNA synthesis were required.

After treatment with FUdR for 12 to 16 hours prior to irradiation it can be seen from Figure 2 that the percentage of cells in mitosis is reduced to approximately 1/4 to 1/6 or less of the untreated control. If TdR were added at the time of irradiation a mitotic delay similar to that in the irradiated control was observed in all cell lines tested. However the data in Table 1 show that addition of TdR at 4, 8 or 12 hours after the irradiation of L-4 or H+ cells, increased the mitotic delays by approximately 4, 8 (L-4 cells only), and 12 hours respectively. For H.Ep.-2 cells, addition of TdR at 4 hours after the irradiation increased the mitotic delay by about 4 hours, but addition of TdR at 8 or 12 hours increased the delay by only about 5 and 7 hours respectively. The evidence indicates then, that at least in L-4 and H+ cells, repair of radiation-induced mitotic inhibition cannot proceed without DNA synthesis. The situation is not so clear-cut in the case of the H.Ep.-2 cells, since it appears that some repair can in fact proceed without DNA synthesis.

The results presented both in Table I and Figure 3 are for a 600 rad dose following a 12 to 16 hour FUdR treatment. Other doses tested were 200, 400 and 800 rads. As would be expected, with lower doses, such as 200 or 400 rads, the mitotic delay is shorter. Consequently the differences between the expected times of mitotic inhibition release for DNA synthesis "required" or "not required" for this release, become small. As a result, one can not from such lower dose experiments, determine with assurance whether DNA synthesis is required for the release of radiation-induced mitotic inhibition. Experiments in which L-4 cells were irradiated with a dose of 800 rads yielded a mitotic delay of at least 10 hours for control cultures. Consequently, there are much greater differences between the expected times of mitotic inhibition release for requirement or non-requirement of DNA synthesis. The results obtained from such experiments support the 600 rad-experiments in indicating that DNA synthesis is required for repair of the mitotic inhibition lesion. Unfortunately, those experiments in which methotrexate was used for the pre-irradiation inhibition of DNA synthesis are not sufficiently complete for comparison with the FUdR experiments.

Although it would appear from the FUdR experiments discussed so far that repair of the mitotic inhibition lesion cannot proceed without DNA synthesis, it also is necessary to consider the effect of the 12-16 hour pre-irradiation FUdR treatment on RNA and protein synthesis. Consequently, biochemical investigations of the effects of FUdR on cellular RNA and protein synthesis, as well as DNA synthesis were undertaken. The results of such investigations on L-4 cells

are presented in Figures 5, 6 and 7. From these figures one can see that although 2×10^{-6} M FUdR starts inhibiting DNA synthesis almost immediately, there does not seem to be a significant inhibition of RNA and protein synthesis until about 14 and 18 hours respectively. Till and Whitmore similarly found no inhibition of RNA synthesis in mouse L-60T cells during a 16 hour treatment with 10^{-7} M FUdR (35). Preliminary experiments with H.Ep.-2 cells indicate that RNA and protein synthesis are not inhibited until Approximately 24 hours after the introduction of 2×10^{-6} M FUdR. Working with HeLa cells, Ruekert and Mueller also found that the accumulation of RNA and protein started falling off after 24 hours of treatment with 10^{-6} M FUdR (56). It has been found with both Ehrlich ascites cells (26) and H.Ep.-1 cells (57) that FUdR at concentrations of 0.75×10^{-8} M to 0.75×10^{-3} M and 4×10^{-7} M respectively do not inhibit the incorporation of orotic acid-C^{14} into RNA uracil or cytosine. Neither is the incorporation of uracil-C^{14} inhibited in ascites cells after treatment with from 0.75×10^{-8} M to 0.75×10^{-3} M FUdR. However, concentrations as low as 10^{-8} M FUdR essentially completely inhibit the conversion of 1.67×10^{-4} M formate-C^{14} into DNA thymine, but not into nucleic acid adenine. In the case of the Ehrlich ascites cells the FUdR was added at the same time as the labeled precursors, whereas the H.Ep.-1 cells were pre-incubated with 4×10^{-7} M FUdR for 24 hours.

Since there does appear to be some inhibition of RNA and protein biosynthesis in mouse L-4 cells after about 14 or 18 hours of treatment with 2×10^{-6} M FUdR, one can not be certain from the results discussed thus far that it was in fact DNA synthesis which was required for the

release of mitotic inhibition. Consequently, it was necessary to conduct some experiments which were similar to those previously carried out, but in which the irradiations were carried out at times when there was inhibition of DNA synthesis, but not of RNA or protein synthesis. If the mitotic delays observed in such experiments were similar to those obtained in the experiments with 12-16 hour FUdR incubation times, one could be more certain that it was in fact DNA synthesis which is required for the release of radiation-induced mitotic inhibition. Results from such an experiment, in which L-4 cells were irradiated at 9 hours after the initiation of FUdR treatment, are shown in Figure 8. The mitotic delays observed are in fact very similar to those in which L cells were irradiated at from 12-16 hours after the initiation of FUdR treatment. Results from an experiment in which L cells were irradiated after a 6 hour FUdR treatment are represented in Figure 9. It appears that a 6 hour pre-irradiation treatment with FUdR is not sufficient to inhibit DNA synthesis to such an extent that no repair of the mitotic inhibition lesion can take place, since the mitotic delays of the cultures other than the control are much shorter than observed in previous experiments. The results, therefore, are interpreted to mean that it is the synthesis of DNA which primarily is required for repair of radiation damage leading to mitotic inhibition. Although RNA and protein syntheses may also be involved, it is unlikely that they are of prime importance. Further evidence for this can be deduced from the findings of Paul and Hagiwara (58) who showed that after FUdR inhibition of RNA and protein syntheses, a lag occurred in the reinstatement of these syntheses after addition of TdR to the cultures.

Thus, if RNA or protein synthesis were required for repair of mitotic inhibition, then after long treatments with FUdR the time between introduction of TdR and release of mitotic inhibition should be greater than the control mitotic delay. This was not observed to be the case, and in fact, the time between introduction of TdR and release of mitotic inhibition was essentially independent of the length of treatment with FUdR.

Since the FUdR experiments have indicated that DNA synthesis is required for the release of mitotic inhibition, one might expect to see unscheduled synthesis of DNA in cells which have been irradiated. Addition of H^3TdR at virtually the same time as irradiation of an asynchronous cell culture should produce label in <u>all</u> cells undertaking post-irradiation DNA synthesis. Thus, after a radiation dose small enough to cause a delay shorter than the length of G_2, autoradiographs of slides prepared during the first 3-3½ hours after the irradiation should show label in mitotic figures if an unscheduled synthesis of DNA had occurred in cells which were in G_2 at the time of irradiation. In the experiments in which H^3TdR (10 c/mM) was added at concentrations of 0.1 or 1.0 μc/ml, no labeling was observed in G_2 cells which had been exposed to 50-150 R. However, when the H^3TdR (6.7 c/mM) was added at a concentration of 10 μc/ml a small amount of label was observed in G_2 cells exposed to 117 R. The results from such an experiment are presented in Table II and Figure 10. These results suggest that DNA synthesis is involved in the repair of the mitotic inhibition lesion. However, the amount of this synthesis probably is small. Examination of a synchronized population of G_2 cells after exposure

to a larger radiation dose probably would show a greater incorporation of label. Unfortunately, synchronous populations of mammalian cells are very difficult to achieve, and with asynchronous cultures it was necessary to limit the dose to a level which would cause a delay not greater than the length of G_2. Consequently the amount of repair and hence the amount of H^3TdR incorporation would be small. Perhaps with the development of more refined labeling techniques the hypothesized phenomenon of radiation-induced unscheduled DNA synthesis will be more accurately described.

Obviously, both in the case of the H^3TdR and the FUdR experiments it would be desirable to repeat such experiments on cell cultures growing in medium which is completely free of thymidine. It may be helpful to do a biochemical analysis of the serum with which the medium is to be supplemented. If TdR were found in the serum, perhaps a more selective dialyzing system could be developed. It would also be interesting to repeat such experiments with microorganisms with well-defined mitotic cycles, which unlike mammalian cells, are capable of growth on simple well-defined media.

Continuation of experiments in which methotrexate is used to inhibit DNA synthesis prior to irradiation could lend support to the experiments presented so far. Also, other inhibitory agents of DNA synthesis such as hydroxyurea (59) could be employed and, if an inhibitor of DNA synthesis could be found which acted immediately, one could easily test the hypothesis that DNA synthesis is required for the release of the radiation-induced mitotic inhibition by the G_2 method initially outlined in the 'Results' section of this treatise.

BIBLIOGRAPHY

1. J. F. Whitfield, R. H. Rixon and T. Youdale Exptl. Cell Res. 27, 143 (1962).
2. J. F. Whitfield and R. H. Rixon Exptl. Cell Res. 27, 154 (1962).
3. J. F. Whitfield, H. Brohee and T. Youdale Exptl. Cell Res. 35, 207 (1964).
4. W. C. Dewey and R. M. Humphrey Rad. Res. 16, 503 (1962).
5. W. C. Dewey and R. M. Humphrey in Cellular Radiation Biology, Williams and Wilkins, Baltimore, 1965.
6. T. T. Puck Revs. of Modern Phys. 31, 433 (1959).
7. T. T. Puck and M. Yamada Rad. Res. 16, 589 (1962).
8. D. O. Schneider and G. F. Whitmore Rad. Res. 18, 286 (1963).
9. L. D. Skarsgard in Radiation Effects in Physics, Chemistry and Biology, Ebert and Howard, North Holland, Amsterdam, 1963.
10. M. M. Elkind and H. Sutton Rad. Res. 13, 556 (1960).
11. G. F. Whitmore, S. Gulyas and J. Botond in Cellular Radiation Biology, Williams and Wilkins, Baltimore, 1965.
12. G. F. Whitmore, J. E. Till and S. Gulyas Rad. Res. 30, 155 (1967).
13. R. A. McGrath Rad. Res. 19, 526 (1963).
14. B. Rao and R. T. Hinegardner Rad. Res. 31, 156 (1965).
15. L. G. Lajtha, R. Oliver and F. Ellis Brit. J. Cancer 8, 367 (1954).
16. R. M. Johns, M.Sc. Thesis, McMaster University, 1967.
17. H. v. Euler and G. v. Hevesy Kgl. Danske Videnskab. Salskab, Biol. Medd. 17(8), 38 (1942).

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

19. D. Billen, R. Hewitt and George Jorgensen Biochim. Biophys. Acta 103, 440 (1965).

20. N. K. Das and M. Alfert Proc. Natl. Acad. Sci. 47, 1 (1961).

21. R. A. McGrath, R. W. Williams and R. B. Setlow Int. J. Rad. Biol. 8, 373 (1964).

22. R. A. McGrath, W. M. Leach and J. G. Carlson Exptl. Cell Res. 37, 39 (1965).

23. D. Petrović, B. Miletić, A. Ferle-Vidović and A. Han Rad. Res. 27, 41 (1966).

24. D. T. Kanazir, O. Ž. Čečuk, B N. Krajčinovič and T. A. Hudnik Bull. Inst. Nucl. Sci. "Boris Kidrich" (Belgrade) 9, 133 (1959).

25. B. Panjevac, G. Ristić and D. Kanazir Proc. 2nd Intern. Conf. Peaceful Uses Atomic Energy, Geneva, 23, 64 (1958).

26. L. Bosch, E. Harbers and C. Heidelberger Cancer Res. 18, 335 (1958).

27. C. W. Young and S. Hodas Science 146, 1172 (1964).

28. R. A. Phillips and L. J. Tolmach Rad. Res. 29, 413 (1966).

29. W. Sinclair Proc. Nat. Acad. Sci. 58, 115 (1967).

30. M. M. Elkind, W. B. Moses and H. Sutton-Gilbert Rad. Res. 31, 156 (1967).

31. D. E. Lea Actions of Radiations on Living Cells, Univ. Press, Cambridge, 1947.

32. D. O. Schneider and R. M. Johns Rad. Res. 28, 657 (1966).

33. M. L. Eidinoff, L. Cheong and M. A. Rich Science 129, 1550 (1959).

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

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

36. A. Fjielde Cancer 8, 845 (1955).

37. C. Norryd and A. Fjielde Cancer Res. 23, 197 (1963).

38. D. K. Ford and G. Yerganian J. Natl. Cancer Inst. 21, 393 (1958).

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

40. H. Eagle Science 130, 432 (1959).

41. T. T. Puck and J. Steffen Biophys. J. 3, 379 (1963).

42. W. C. Schneider J. Biol. Chem. 161, 293 (1945).

43. K. Burton Biochem. J. 62, 315 (1956).

44. Z. Dische Mikrochemie 8 4 (1930).

45. H. G. Albaum and W. W. Umbreit J. Biol. Chem. 167, 369 (1947).

46. Z. Mejbaum Physiol. Chem. 258, 117 (1939).

47. O. H. Lowry, N. J. Rosenbrough, A. L. Farr and R. J. Randall J. Biol. Chem. 193, 265 (1951).

48. V. Defendi and L. A. Manson Nature 198, 359 (1963).

49. C. P. Stanners and J. E. Till Biochim. Biophys. Acta 37, 406 (1960).

50. J. H. Priest, J. E. Heady and R. E. Priest J. Nat'l Cancer Inst. 36, 61 (1967).

51. T. Terasima and L. J. Tolmach Biophys. J. 3, 11 (1963).

52. S. Mak and J. E. Till Rad Res. 20, 600 (1963).

53. R. E. Handschumacher and A. D. Welch in The Nucleic Acids Vol. III, E. Chargaff and J. N. Davidson, Academic Press Inc., N. Y., 1960.

54. T. C. Hsu, R. M. Humphrey and C. E. Somers J. Natl. Cancer Inst. 32, 839 (1964).

55. M. L. Eidinoff and M. A. Rich Cancer Res. 19, 521 (1959).

56. R. R. Rueckert and G. C. Mueller Cancer Res. 20, 1584 (1960).

57. L. Cheong, M. A. Rich and M. L. Edinoff Cancer Res. 20, 1602 (1960).

58. J. Paul and A. Hagiwara Biochim. Biophys. Acta 61, 243 (1962).

59. S. E. Pfeiffer and L. J. Tolmach Cancer Res. 27, 124 (1967).