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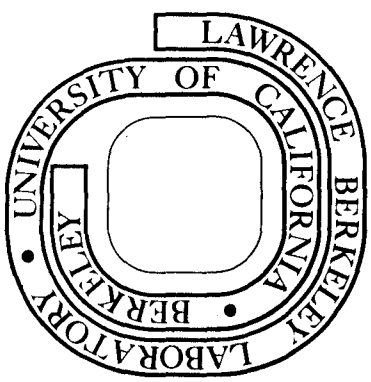

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Bromodeoxyuridine Induced Mutations in Synchronous Chinese Hamster Cells: Temporal Induction of 6-Thioguanine and Ouabain Resistance During DNA Replication

by

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ABSTRACT

Mutations were induced in synchronous Chinese hamster cells by exposure to bromodeoxyuridine (BUdR) for one hour pulses. There was a very pronounced temporal dependence during the first half of the DNA synthesis period for the induction of 6-thioguanine (6TG) and Ouabain (OUA) resistance. No mutants above background were induced by exposure to BUdR in G_1 and G_2 cells, and very few mutants were induced in the latter part of the DNA synthesis period. The peak for the induction of 6TG resistance occurs at about 2 hours in the DNA synthesis period followed one hour later by the peak for the induction of OUA resistance. Both peaks occur before the time of maximum incorporation of BUdR into DNA. This sequence of induced mutation to 6TG and OUA resistance was found in cell lines synchronized using hydroxyurea resynchronization and in cells synchronized mechanically under optimum growth conditions without the use of drugs.

These results suggest that the mutagenesis by BUdR is associated with at least two genes which replicate at 2 hours and 3 hours in the DNA synthesis period.

Mammalian cells replicate their several meters of DNA under culture conditions in about 6-8 hours. Replicating units of the DNA replicate in two directions at a rate of about 1 micron per minute, (Huberman and Riggs, 1968). The number of replicating units replicating at one minute intervals is, on the average, about 10^4, (Painter and Schafer, 1969; Painter et al., 1966). The time of replication of regions of chromosomes has been shown by many researchers to follow a temporal pattern in the DNA synthesis period. (Examples: Stubblefield, 1975; Mukherjee et al., 1968). Generally euchromatic DNA replicates early and heterochromatic and centromeric regions replicate later, (Comings, 1972; Utakoji & Hsu, 1965; Stubblefield, 1975). There are selected intervals in the DNA synthesis period when specialized DNA replicates, e.g. satellite DNA and nucleolus organizer DNA, (Stambrock, 1974; Amaldi et al., 1969; Bostock & Prescott, 1971; Flam et al., 1971). The time of replication of DNA appears to be related to organ differentiation, (Utakoji & Hsu, 1965), and there appears to be a specific time in the synthesis period when SV40 DNA is replicated, (Balazs et al., 1973).

The association of a particular cellular function with a region of the DNA can be accomplished by damaging a region in synchronous cells by incorporating BUdR, or related agents, like iododeoxyuridine (IUdR), tritiated thymidine (^{3}H-TdR), tritiated idodeoxyuridine (^{3}H-IUdR), tritiated bromodeoxyuridine (6-^{3}H-BUdR), and ^{125}I-iododeoxyuridine (^{125}I-IUdR), into the DNA in short pulses during DNA synthesis. Inducing a change

using bromodeoxyuridine (BUdR) or other thymidine analogues is usually a proof of function associated with damage to a particular DNA region although the mechanism for the BUdR damage is not always clear. Once BUdR is incorporated into DNA, damage can be induced by exposure to visible light, (Chu et al., 1972). These methods have been exploited to suggest that specific genes replicate at specific times during the cell cycle (Suzuki & Okada, 1975; Aebersold & Burki, 1976; and Kasupski & Mukherjee, 1977), that 'critical DNA' damage occurs in late replication DNA (Burki, 1974 & 1976), and that C virus and Epstein Barr viruses are activated at specified intervals within the S period, (Besmer et al., 1974; Hampar et al., 1973). The work of Suzuki and Okada (1975), and Aebersold and Burki, (1976) suggests that the early DNA synthesis period is critical for mutagenesis. However, the results do not distinguish between a "hot time" in the cell cycle for mutagenesis and individual gene duplication, as suggested by the suppression of enzyme levels in mouse cells as a function of the time of incorporation of BUdR (Kasupski & Mukherjee, 1977).

If the peak of BUdR induced mutagenesis (Suzuki and Okada, 1975; and Aebersold and Burki, 1976) were due to a "hot time" for mutagenesis in the cell cycle, one should find that the induction of drug resistance related to two different enzymes should occur at the same time in the cell cycle. If different times for maximum mutagenesis were found this would suggest that the genes associated with the induction of drug resistance replicated at different times within the DNA synthe-

sis period. The induction of two different peaks for the different loci would also be evidence against other hypotheses to explain the data, e.g. thymidine pool effects, epigenetic mechanisms, and mitochondrial DNA replication. [Hamster cell mitochondria appear and disappear at different times in the cell cycle, (Ross & Mel, 1972) and in other species the mDNA replicates in S and G2, (Koch & Stochstad, 1967; Bosman, 1971; Pica-Mattoccia and Attardi; 1972) although no cyclic variation has also been seen after double thymidine block synchrony, (Robinowitz & Swift, 1970)].

The two mutational systems used in this series of experiments were the induction of resistance to 6TG and the induction of resistance to ouabain. Much work has been completed on the isolation and characterization of cells resistant to purine analogues with particular emphasis on the purine salvage pathway, (Carver et al., 1976; Arlett et al., 1975; Thacker et al., 1976; Fox et al., 1976; [for review see Siminovitch, L., 1976]). A majority of the clones resistant to 6TG exhibit a low level of hypoxanthine-guanine phosphoribosyltransferase (HPRT) activity, (Gillin et al., 1972) although some clones do not, (Aebersold, 1975). The gene for HPRT is X linked in humans, (McKusick, 1973) and in Chinese hamster cells, (Westerveld, 1972) and variant cells resistant to purine analogues appear to be due to alterations or loss of the gene coding for HPRT. It is important to note that a loss of the HPRT enzyme is not a lethal event. Mutations to 6TG resistance can be induced by X-rays, UV, alkylating carcinogens, and incorpora-

ted 5-methyl^{3}H-TdR, (Thacker & Cox, 1975; Cleaver, 1977; Bridges & Huckle, 1970; Hsie et al., 1975; and Van Zeeland et al., 1976).

Resistance to ouabain is characterized by alteration of the $Na^{+}K^{+}$ activated ATPase of the cell membrane, (Baker et al., 1974) so that this enzyme can no longer bind the drug but can still maintain active transport. The complete loss of this enzyme is a lethal event, and only those cells with functional altered enzymes not binding ouabain can be seen by the assay. Thus point mutations are liable to be the basis of ouabain resistance. Resistance to ouabain may be induced by exposure to UV and to alkylating carcinogens, (Baker et al., 1974) and exposure to BUdR, (Aebersold, 1975), but not by X-rays, or incorporated tritium, (Cleaver, 1977). These results suggest that although these two mutagenic tests are mutagen specific, they could be used for BUdR mutagenesis to test the hypotheses above.

The mechanism for BUdR mutagenesis is unkown at this time in mammalian cells. However, in bacteria THE EFFECTS of BUdR are not proportional to the amount of BUdR incorporated into DNA and can be directly related to mismatch repair processes which are independent of the recA and uvrA gene functions and dependent on uvrD and uvrE functions, (Rydberg, 1977). It has been shown that the mutagenic effects of BUdR incorporated into DNA are not proportional to the concentration of BUdR in Chinese hamster cells, (Aebersold, 1976) suggesting an allosteric effect on DNA synthesis. It is therefore reasonable to have two

working hypothesis at this time: BUdR induced mutations occur 1) as a result of allosteric alteration of DNA synthesis at the site of replicating DNA or, 2) as a result of mutations produced by mismatch repair of DNA containing BUdR. Both of these hypotheses require that the mutation be a result of the presence of BUdR during the replication of the gene or genes responsible for the mutant phenotype.

Materials & Methods

Cell Lines and Culturing Procedures

The CHO cell line was derived from primary Chinese hamster ovary tissue by Tjio and Puck (1958). One CHO cell line used in our laboratory was obtained from S. Wolff (UC San Francisco, Lab of Radiobiology) in 1973, and aliquots have been frozen and kept at liquid nitrogen temperatures since then. The other CHO cell line was received from L. Kapp and R. Klevecz, City of Hope Medical Center, Duarte, CA., and had been selected for use on the cell cycle analyzer (Talandic Research Corporation). Stocks of this second CHO cell line which we call CHO-kk have been kept frozen in our laboratory since 1976. Fresh cultures are started about 4 times a year to insure that the genetic changes in the cell lines during growth at 37° are minimal and reproducible from year to year.

These cell lines have been shown to be free of PPLO and have a modal chromosome number of 21, as others have reported. The G banded karyotype of the CHO-kk cells has been determined and shows four abnormal autosomes and only one complete X chromosome, (Burki, unpublished). The population doubling time of the cells is about 12 hours under optimum conditions with a 4 hour G1 period, 6 hour S period, and a G2 plus M period of 2 hours.

The CHO cell lines are grown at 37° in a CO_2 incubator or in closed tissue culture flasks at 37°C in McCoy's 5A media supplemented with 15% fetal calf serum and 100 units per ml of penicillin and 100 micrograms per ml of streptomycin. The CHO-kk cells are

grown in the same media but with the addition of 1mM HEPES buffer to slow pH changes during experiments.

Synchronization Methods

1) CHO cells

Cells which had been continuously subcultured in logarithmic growth for several days were plated into 250cc tissue culture flasks at 10^6 cells per flask in 10cc of media. The cells were permitted to grow for 30 hours and then gently shaken for 10 seconds at two shakes per second. (Care must be taken in growing these cells not to accidently cause colony abortions which will affect plating efficiencies). A typical yield is about 10^5 cells per flask with a mitotic index of greater than 90%. In experiments where the cells were resynchronized with hydroxyurea, hydroxyurea (1mM) was added immediately after shake off. This drug was then removed after 11 hours by washing 2X with prewarmed Pucks saline A. When the cells began DNA synthesis in fresh warm media, BUdR was added at 0.5mM according to the design of each experiment.

2) CHO-kk cells

Cells were grown for several days in logarithmic growth phase in roller bottles turned at 0.5 rev/min. They were subcultured into one or more roller bottles at 2-3 X 10^7 cells per bottle. After 48 hours the cells were synchronized in a 37° room using a Cell Cycle Analyzer apparatus (Talandic Research Corp. Duarte, CA). This apparatus is patterned after the instrument developed by Klevecz (1972). The mitotic cells were usually shaken off at one hour intervals at the following speeds: 54 minutes at 0.5 RPM, 3 minutes at 180 RPM, 2 minutes for cell

collection, and 1 minute for media replacement in the roller bottle. This apparatus thus gives continuous cohorts of cells spaced in these experiments one hour apart in cycle time. The typical yield per roller bottle is 2 X 10^6 cells in very early G1. Greater than 90% of the cells at harvest appear as small doublets. These cells are permitted to attach to plastic tissue culture flasks and labelled with BUdR or thymidine according to each experimental format.

Cell Life Cycle Progress Analysis After Mitotic Detachment

1) CHO cells

Three methods were used to monitor the cell progress through the cell cycle. a) The cell number was determined as a function of time using a Coulter Counter Model F; b) The toxicity of a second dose of 1mM hydroxyurea to the cells as a function of time; and c) the uptake of pulses of 3H-BUdR at low specific activity. The acid insoluble radioactivity was determined as below.

2) CHO-kk cells

Two additional techniques were available for these cells, volume spectroscopy and flow cytometry. a) Cell volume spectroscopy The modal size and the volume distribution of the trypsinized synchronous cells at different positions in the cell cycle were determined using a Coulter Counter Model ZBI matched to a Coulter Channelyzer. This system was calibrated using 10 micron spheres provided by the Coulter company and ragweed

pollen. An X-Y plotter was used to graphically determine the coefficient of variation of the distribution of pulses created by the cell populations as a function of the cell cycle time. The volume of the cells appeared to increase almost linearly until the cells divided after approximately 12 hours. b) Flow Cytometry (Flow Microfluorimetry) Cells at different times after mitotic detachment were collected by gentle centrifugation and fixed at 0°C using a fixitive containing 3 parts 200 mM $MgCl_2$ + 1 part absolute ethyl alcohol and kept at 4°C. They were then stained with chromomycin A3 (100 µgm/ml), (Cal Biochemical), (Toby and Crissman, 1975) for at least one hour. Analysis of the fluorescence was made using an instrument according to the design of Steinkamp et al. (1973) and described by Hawkes and Bartholomew (1977). In these experiments the wavelength used for excitation was 4570 Å at a laser power of 0.6 watts with approximately 500 cells per second intersecting the beam from the Argon-Ion laser. The coefficient of variation was calculated by standard methods. Although the coefficient of variation has been as small as 2.5%, it is usually 4% in non S cells. (See text). c) Uptake of Tritiated BUdR. Usually 10^6 cells were washed 3 times with cold 10% TCA and then kept at 90° centigrade for 1 hour to hydrolyze the cell DNA for a determination of the amount of precursor incorporated into DNA at different times. The radioactive mixture was added to PCS solubilizer for liquid scintillation counting, (Amersham : Searle). The absolute counting efficiency for the samples was determined by using

tritium quench standards and the channels ratio method.

Selection for Drug Resistance

Clones resistant to 5 μgm/ml of 6 thioguanine or 3mM ouabain were selected for after an expression time of 5 days had passed. Previous experiments had shown that this was an adequate expression time for 6TG resistance. Although more rapid expression times are found for ouabain resistance, it was convenient to use the same expression time as for 6TG.

It is important to note that in the usual experiment the cells were kept in log stage for the entire expression period. The usual procedure was to initially use an aliquot of cells of about 2×10^5 cells and permit growth in a large tissue culture flask for 2.5-3.0 days and then subculture to 2×10^5-10^6 cells again in order to grow sufficient cells for the challenge on the fifth day. Growth curves monitoring the growth of cells after these treatments showed that at least 7-10 population times occurred under the experimental conditions.

The plating efficiency of cells 5 days after synchrony and BUdR exposure was indistinguishable from stock CHO cells. BUdR is more toxic to early S phase cells than to late S phase cells (Kajiwara and Mueller, 1964; Aebersold, 1976).

After eight days in the selective drug the number of clones was determined by counting the number of colonies stained with 1% methylene blue. Recent experiments where designed to insure that at least 100 clones above background were obtained after treatment at peak times for BUdR mutagenesis, to insure 10% statistical

reliability at the peak time of mutagenesis. This usually means that 5 plates must be seeded at 2×10^6 and 2×10^5 cells per plate for the OUA and 6TG experiments respectively.

RESULTS

In initial experiments with synchronous CHO cells, the detached population was about equally divided between small volume G1 cells and large cells completing mitosis. These early shake off procedures involve shaking off cells at room temperature which caused an extended G1 period as demonstrated by pulse labeling the cells with tritiated thymidine (Figure 1). This data demonstrated that DNA synthesis does not begin until 6-8 hours after shake off. Since during this period of time the synchrony decayed considerably, cells were resynchronized near the early S period interface by adding 1mM hydroxyurea for 11 hours after mitotic detachment. Hydroxyurea is known to inhibit DNA synthesis almost completely in Chinese hamster cells, (Sinclair, 1967; Burki, 1976), although there is about 5% of the DNA synthesized in the presence of hydroxyurea, (Meyn et al., 1975; and Walters et al., 1976). After release from the hydroxyurea block, the cells proceed rapidly through the S period (Figure 2). The toxicity of a second exposure to hydroxyurea treatment shows that the cells enter the G2 period about 5.5-6.0 hours after the hydroxyurea removal. This is a slight shortening of the S period in Chinese hamster cells, (Sinclair, 1967). (Compare figure 4A and figure 10A). The time when the cells enter the next G1 period after the hydroxyurea resynchrony is seen on the right side of Figure 2 to be about 8 hours; it is also seen that about 90% of the cells divide by 10 hours. The uptake of tritiated BUdR into DNA in one hour

pulses after release from hydroxyurea is seen in Figure 4A.

Hydroxyurea induced resynchronization enabled us to perform the experiment in which cells were mutagenized by BUdR exposure for each hour during the DNA synthesis period. The results are shown in Figure 3. The maximum peak for induction of 6TG resistance by BUdR occurs between the first and the second hour while the induction of resistance to ouabain occurs one hour later. The large variation in the ouabain peak was due to the fact that the peak position in one experiment was slightly later. It is important to note that both peaks occur in the DNA that replicates in the early part of the DNA synthesis period. In figure 4A the peak for the incorporation of tritiated BUdR is between the third and the fourth hour after release from hydroxyurea. If the data in figure 3 are normalized to the amount of 3H BUdR incorporated into DNA within the time that the mutagenesis occurred and the peak of mutagenesis is denoted by 1.0, the relative efficiency for the induction of mutation as a function of the time in the DNA synthesis period can be obtained. A plot of the data normalized in this fashion is given in figure 4B. Here it is seen that the mutagenesis peaks are symmetrical and occur one hour apart when corrected to the amount of BUdR incorporated into the DNA.

It became important at this point in our experiments to insure that the results were not associated with the method of synchrony used, and that the other stages of the cycle be exposed to BUdR to eliminate the possiblity that BUdR could

induce mutations by being present during non DNA synthesis phases. If BUdR induced mutations outside the DNA synthesis period, then the results could be associated with effects other than BUdR incorporation into DNA or its presense during replication. The elimination of possible hydroxyurea effects is very important since it is established that 5% of the DNA is replicated in the presense of hydroxyurea, thus distorting the order of replication of the replication units in the nucleus, although hydroxyurea is far superior to the use of thymidine blocking where 30% of the DNA replicates, (Meyn et al., 1975). Therefore experiments with a completely selective system for synchrony (Klevecz, 1972) would eliminate possible artifacts from the synchronous Chinese hamster cell system.

CHO-kk cells were detached during mitosis from glass roller bottles using the cell cycle analyzer developed by Klevecz (1972) and used in human cell synchrony (Kapp & Klevecz, 1973). The cells were treated as in each experimental protocol and then the volume of the cells determined using a Coulter-channelyzer system equipped with an X-Y plotter. The peak of the size distribution was determined. The coefficient of variation of electronic sizing data of this type is usually about $\pm$ 14%. This peak in the distribution corresponding to the modal cell volume was found in a particular channel corresponding to the time of the cell cohort in the cell cycle. The data from a typical experiment are given in figure 5. In other experiments not reported here the synchronous change in cell volume as a

function of time continued for at least the next generation with a very slow rate of decay of the degree of synchrony in the cell population.

The progress of the synchronous CHO-kk cells was also followed through the cell cycle using the flow cytometric method (flowmicrofluorimetry) after staining fixed cells from different cohorts with chromomycin A3 (Cal Biochem); the data is shown in figure 6. The first panel shows the initial distribution of fluorescence of the population one hour after mitotic detachment. The amount of fluorescence corresponds to the amount of dye bound by the DNA in the cell. If this dye binding is stochiometric for the cellular DNA content under these conditions, then this first panel shows a population which is 99% in the G1 stage with one unit of DNA per cell. The coefficient of variation of this group is about 4%. The cells proceed through the S period as seen in the panels corresponding to 4, 5.5, and 7 hours respectively. The coefficient of variation characteristically increases through the S period to 15% during mid S in these cells. Most of the cells are in G2 or mitosis after 10.0 hours and the population is one half divided at 11.5 hours. After 13 hours the cells are again in the G1 stage of the cycle although the coefficient of variation has increased slightly to about 6% in the main peak and a small portion (5%) of the cells appear to be moving through the cycle more slowly than most of the population. This data may be summarized in terms of the peak channel corresponding to the DNA content and the time in

the cell cycle since mitotic detachment. In the experiment shown in figure 7 the cell life cycle corresponds to a G1 period of 4 hours, an S period of about 6 hours, and a G2 + M period of about 1.5 hours. The exact life cycle parameters may vary from experiment to experiment, but, for cells grown in the dark or in yellow fluorescent light, the population doubling time was between 11.5 and 12.5 hours.

Care must be taken to avoid exposure of the cells or the growth medium to normal white laboratory fluorescent light, (Jostes et al., 1977; Bradley & Sharkey, 1977), since along with induction of mutations the population doubling time of the cells may be extended due in part to the killing of cells after fluorescent light exposure. Gold fluorescent light exposure does not cause these effects, (Burki & Lam, 1977).

For mutagenesis of synchronized cells some of the various cell population cohorts were exposed to one hour pulses of 0.5mM BUdR or tritiated BUdR,1μC/ml at 0.5mM. After labelling and washing, the cell number, cell volume, DNA content, and uptake of tritiated BUdR were determined. The cells were subcultured after 3 days and challanged after 5 days to 5μgm/ml of 6TG or 3mM ouabain. Visible colonies were counted after 8 days growth in the selective media. The results for controls exposed to 1 hour pulses of 0.5mM thymidine are shown in figure 8. The average mutation rate for the whole cycle given by the dotted line in figure 8 was $0.33 \times 10^{-5} \pm 0.3 \times 10^{-5}$ for 6TG and $0.5 \times 10^{-6} \pm 0.5 \times 10^{-6}$ for ouabain. The individual means for

drug resistance in the cycle were not significantly different from the cycle mean. The induction of mutations by exposure to BUdR for one hour pulses is given in figure 9. There is a peak for the induction of mutations to 6TG resistance which is approximately 50 times the background level at 6 hours after mitotic detachment. Ouabain resistance is induced maximally at 7 hours after mitotic detachment at a rate 25 times background. The peaks are separated by about one hour in the DNA synthesis period as in the experiments using hydroxyurea induced resynchrony. These peaks do not correspond to the peak time for incorporation of tritiated BUdR into DNA, which occurs at about 9 hours (Figure 10A). For convenience, the S period is taken as the time when there is significant uptake of 6-^{3}H-BUdR (5% of peak value) since some cells begin synthesis slightly earlier than others. Induction of mutation normalized for the amount of BUdR incorporated into the DNA at each time in the cycle is shown in figure 10B. There are peaks for the induction of mutations within the DNA synthesis period at 2 hours for 6TG and at 3 hours for ouabain. These results are very similar to the results with CHO cells in figure 4B. There are also some smaller peaks above background in the S period which appear to be significant and may be related to 5% of the population which transverses the cycle at a slower rate. There do not appear to be any significant peaks for BUdR mutagenesis in the G1 period.

DISCUSSION

The induction of drug resistance due to exposure of cells to BUdR during DNA synthesis occurs at specific times in the first half of the DNA synthesis period. The induction of 6TG resistance is maximum at about 2 hours after the initiation of DNA synthesis; one hour later there is a peak in the induction of ouabain resistance. These peaks occur in cells synchronized with or without hydroxyurea resynchrony. There does not appear to be any induction of drug resistance for cells which are exposed to BUdR in G1, G2+M, or in the late S period. These data constitute strong evidence that the induction of drug resistance is due to BUdR induced mutations in the gene or genes associated with the production of the HPRT enzyme and the Na^+ K^+ ATPase enzyme. Hypotheses not supported by this data are 1) that there are "hot times" for mutagenesis in the cell cycle in the early S period, 2) that epigenetic events or mitochondial DNA mutations are responsible, or 3) that nuclear pools of TdR are the cause of these results.

It is not surprising that nuclear genes coding for functions associated with resistance to these two drugs are replicated in the first half of the DNA synthesis period. It has been long known that the euchromatic DNA replicates earlier in the DNA synthesis period than the heterochromatic, centrometic, and pericentromeric DNA. These experiments place the two regions associated with 6TG and OUA resistance near the middle of the euchromatic DNA replication sequence.

Resistance to 6TG is associated with a change in the DNA found on the X chromosome, is recessive, and can be induced by

a variety of molecular and chromosome lesions. Ouabain resistance on the other hand,is associated with point mutations which lead to enzymatic changes in the cell membrane associated ATPase. These point mutations probably occur in one of the autosomes of the Chinese hamster cell and are codominant. However, the ratios of induced to background mutations are similar for 6TG and OUA. This suggests that 6TG and OUA resistance are induced by BUdR by similar genetic events, probably point mutations.

Although the mechanism for the induction of mutations by BUdR in mammalian cells is not yet known, the induction rate as a function of BUdR concentration is not linear, (Aebersold, 1976). This nonlinearity is similar to results seen in bacteria, (Rydberg, 1977). In bacteria, careful studies with repair efficient stains suggest that "mismatch repair" occurs leading to base transitions in the DNA after the incorporation of BUdR. This may be a good preliminary hypothesis to test for the mechanism of mutagenesis in mammalian cells, although allosteric alteration of DNA synthesis by BUdR is also possible (Aebersold, 1977). Thus, we propose that the mutations to drug resistance above are related to mismatch repair or allosteric alteration of DNA synthesis leading to transitions in the DNA within regions of DNA replicated during the pulse of BUdR.

We originally proposed to study the functional organization of the nucleus using incorporated radioisotopes and related agents with the hope of being able to study intranuclear regions associated with specific cell functions. By damaging

these regions we hypothesized that we could study such phenomena as intranuclear repair, mutagenesis, and intranuclear toxicity. The results reported here together with previous reports of 'critical DNA' within the nucleus suggest that intranuclear studies are feasible. The data in this paper suggest that temporal replication of genes can be demonstrated in Chinese hamster cells.

FIGURE CAPTIONS

Figure 1

Initiation of DNA synthesis following manual shake off of CHO cells. Replicate cultures were established at 41,000 cells/plate and sequentially exposed to 0.2 uCi/ml of tritiated thymidine for one hour intervals.

Figure 2

Analysis of the synchrony of CHO cells after resynchrony with hydroxyurea. Triangles show the ratio of cell number at subsequent times to cell number at rinsing off the hydroxyurea from replicate cultures in two experiments. The data are the averages from shake offs yielding 113,000 and 84,000 cells/plate. Circles show the survival of cells after exposure to hydroxyurea a second time for six hours commencing at the times indicated. Innoculations were at 200 cells per plate and the data are the average and standard deviation from two experiments.

Figure 3

6Thioguanine and Ouabain resistance induced by BUdR in CHO cells. Replicate cultures were labelled with 0.5 mM BUdR during different hours of the DNA synthesis period. After an expression time of 5 days, sister subcultures were exposed for 8 days to either 5µgm/ml of 6TG or 3mM ouabain.
Left: The average of four experiments showing induction of 6TG resistance; error bars are the standard deviation of the mean of four experiments.

Right: Average of three experiments showing the induction of ouabain resistance; error bars show the standard deviation of the mean from the three experiments.

Figure 4

BUdR Incorporation and Normalized Efficiency of Induction of 6TG and OUA Resistance.

Left: The incorporation of tritiated bromodeoxyurdine into DNA during sequential one hour pulses of 1μCi/ml of 3H-BUdR at 0.1mM BUdR. The cells were trypsinized eight hours after rinsing off the hydroxyurea and the absolute amount of tritium incorporated determined as in the procedures section.

Right: Normalized induction of 6TG (·----·) and OUA (o——o) resistance. The data in figure three were normalized to the amount of BUdR incorporated into the DNA at that time. The peak of mutagenesis is arbitrarily called an efficiency of 1.0.

Figure 5

Cell Volume of Cell Cohorts of CHO-kk cells after automatic synchrony. Individual cohort cultures at various times from the time of shake off were trypsinized and aliquots of the cells were counted on a Model ZBI Coulter counter equipped with a 100 x 120 micron orifice. The distribution of pulses proportional to cell volume was analyzed using a 100 channel analyzer (Coulter Channelyzer) and the peak channel was recorded for each sample of cells.

Figure 6

Flowcytofluorimetric Data for Cell Cohorts of CHO-kk cells. Individual cohort cultures at various times after shakeoff were trypsinized and aliquots of fixed cells stained with chromomycin A3 were analyzed using the FCM method. The data from a typical experiment for the distribution of fluorescence (proportional to DNA content) is given on the 9 pannels. The maximum number of cells in each channel analyzed was 4,000. The numbers in each panel are the times after detachment. The abscissa is the amount of fluorescence ecorded.

Figure 7

Peak Channel Fluorescence as a Function of Time after Mitotic Selection. The data of Figure 7 can be used to determine the average DNA in the cohorts (proportional to fluorescence) as a function of time after selective detachment of the cells. The mean and the coefficient of variation of the mean is given in the graph. This data combined with data on the incorporation of tritiated BUdR (Figure 10 Left panel) enables the life cycle parameters of the cells to be ascertained.

Figure 8

Background Mutagenesis in Synchronous CHO-kk cells in the Dark. Synchronized cells were exposed to one hour pulses of TdR 0.5mM and grown in a dark 37°C walk-in incubator. After 5 days the cells were challanged to 6TG or OUA as described in experi-

mental procedures. The dotted lines are the average number of induced drug resistant clones throughout the complete cell cycle.

Figure 9

BUdR Induced Mutagenesis in Synchronous CHO-kk cells in the Dark. Synchronized cells were exposed to 1 hour pulses of 0.5 mM BUdR under optimum conditions. After an expression time of five days, cells were challanged by exposure to either 6TG or OUA as described in experimental procedures. The values are the mean and standard deviation from one experiment. Background levels are not yet subtracted in this graph. Please note that the vertical scale is different than that of figure 8.

Figure 10

Left: Incorporation of 3H-BUdR into DNA in CHO-kk cells. Cohorts of cells were permitted to incorporate 3H-BUdR at 0.5mM and 1μCi/ml, under the same conditions in which mutations were induced.

Right: Normalized Mutation Frequency for the induction of 6TG resistant clones (dotted line closed circles) or ouabain resistant clones (solid line open circles). Normalization is as in figure 4 with 4 hours assumed to be the initiation of DNA synthesis.

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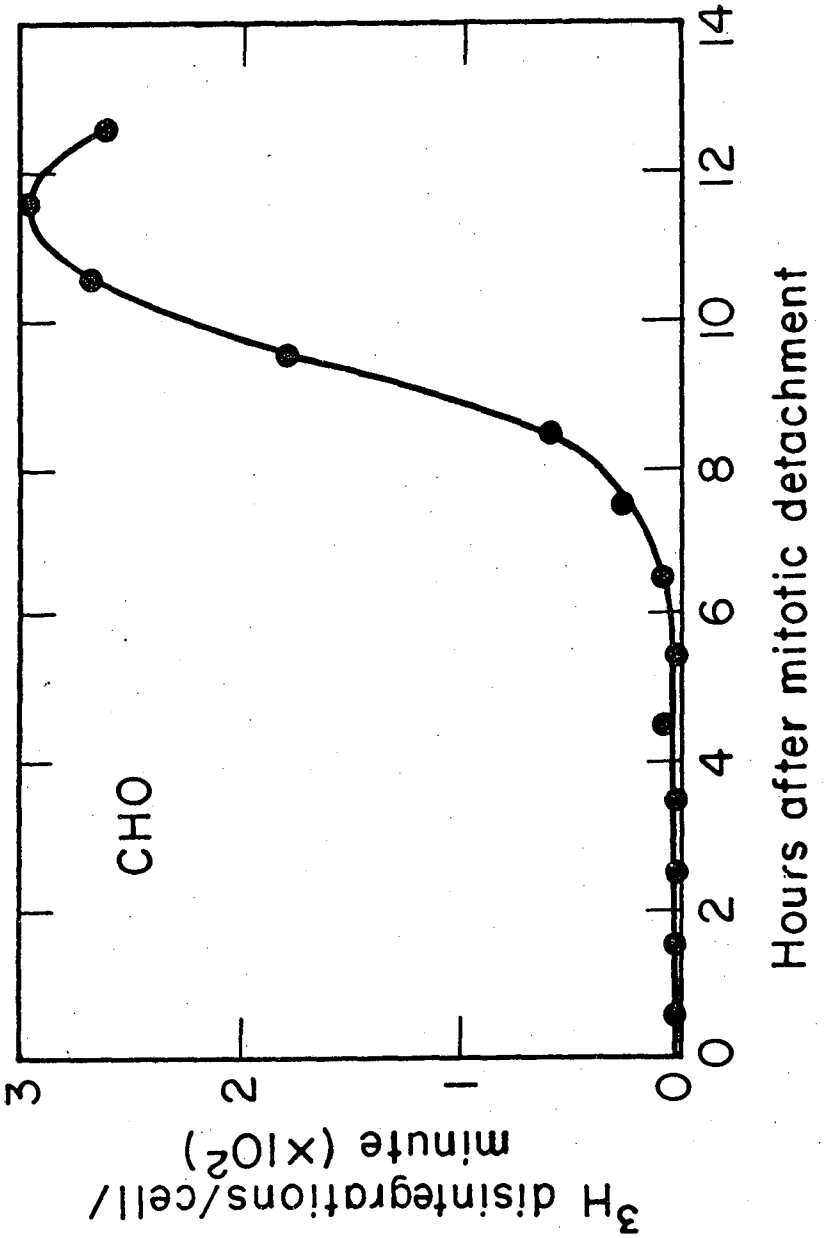

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Fig. 1

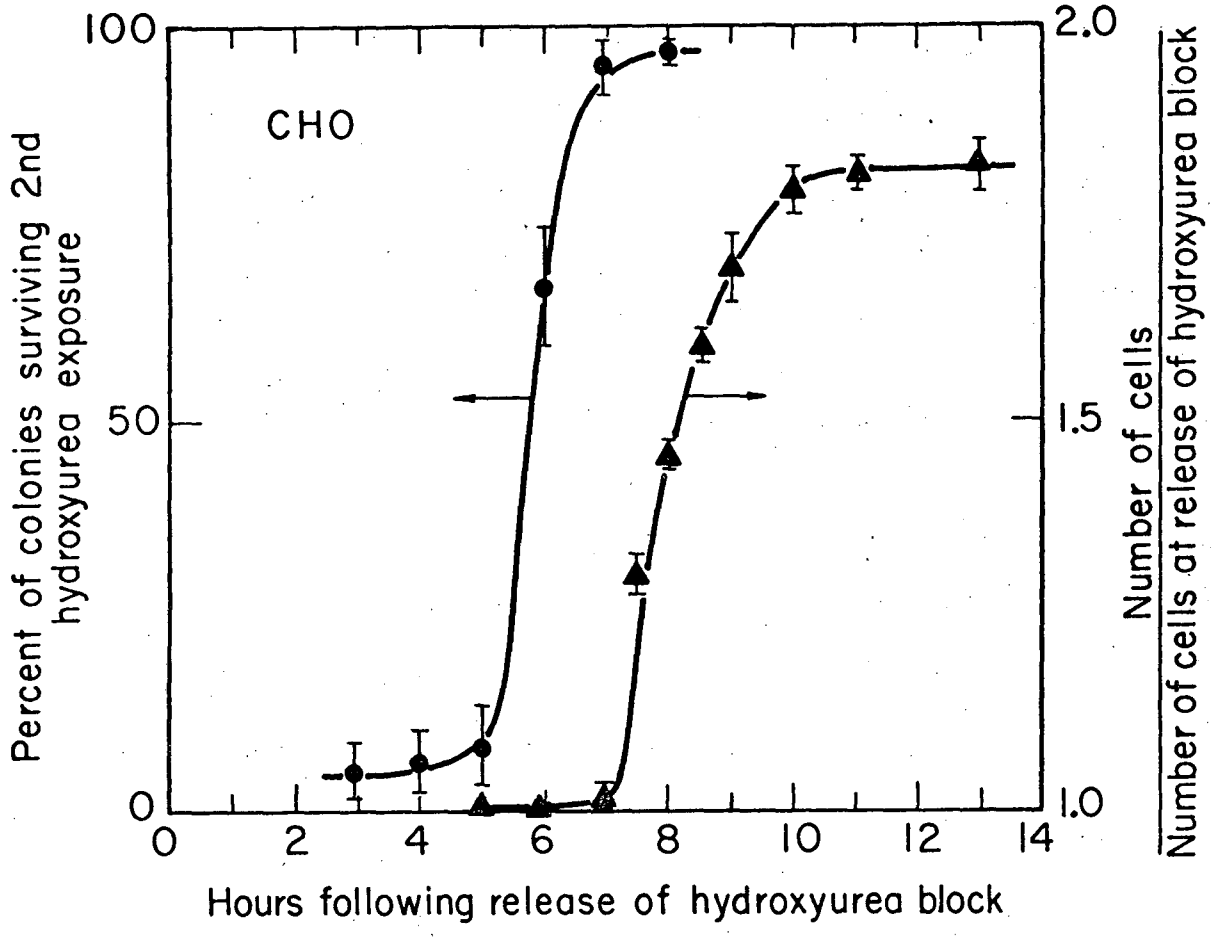

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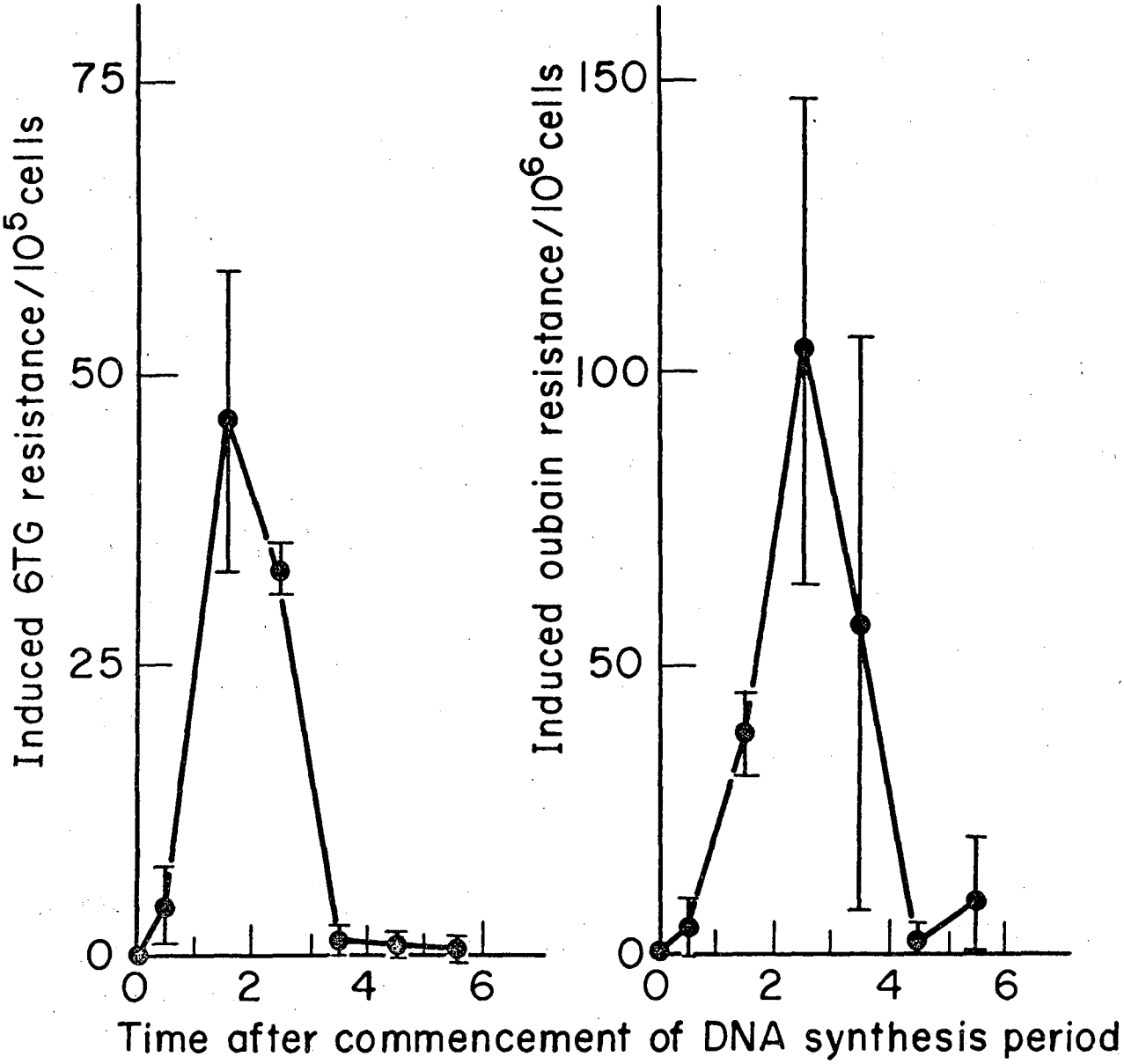
Fig. 2



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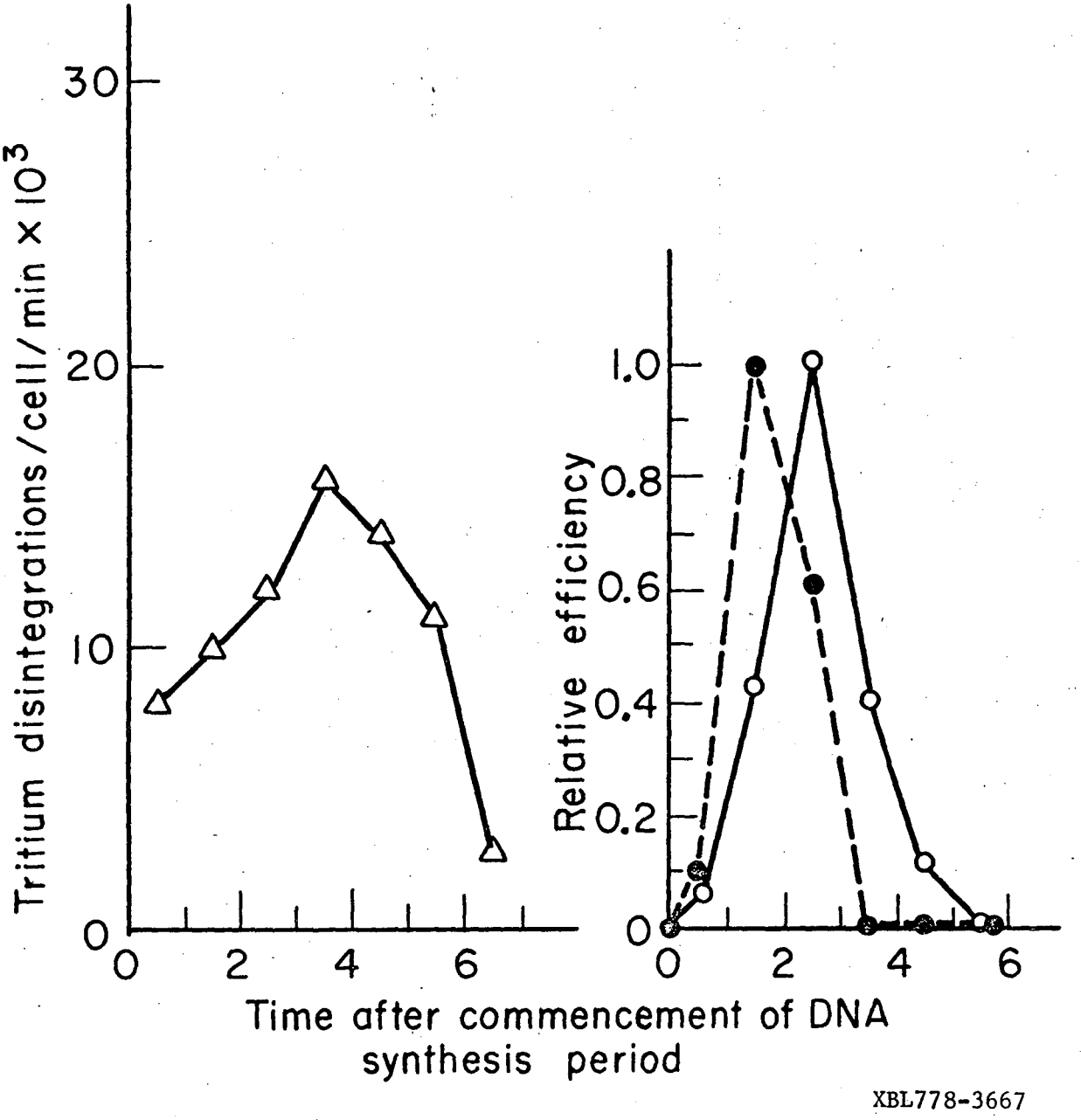
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20
10
0
0
2
4
6
Relative efficiency
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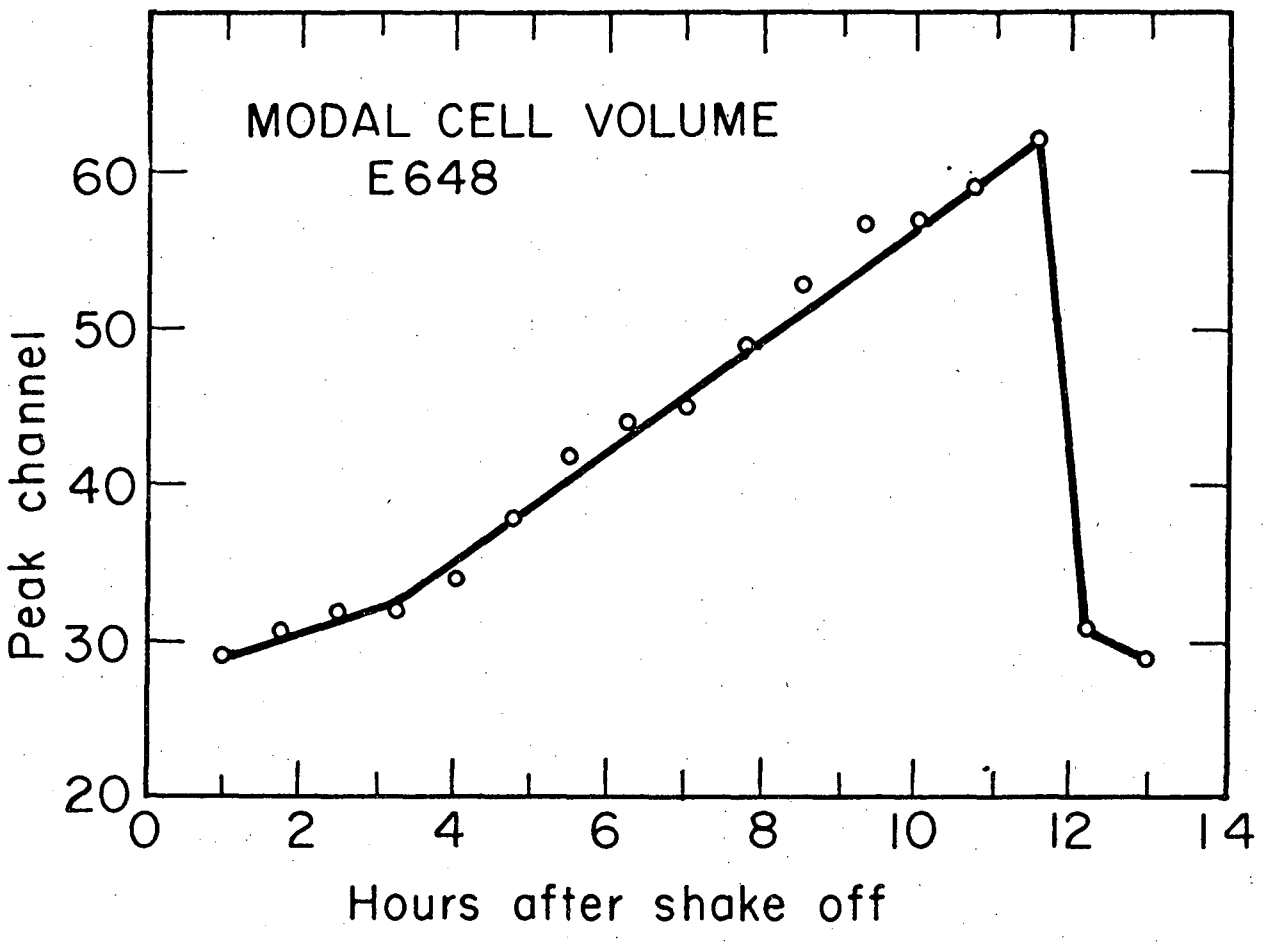
0.4
0.2
0
0
2
4
6
Time after commencement of DNA synthesis period
XBL778-3667

Fig. 4



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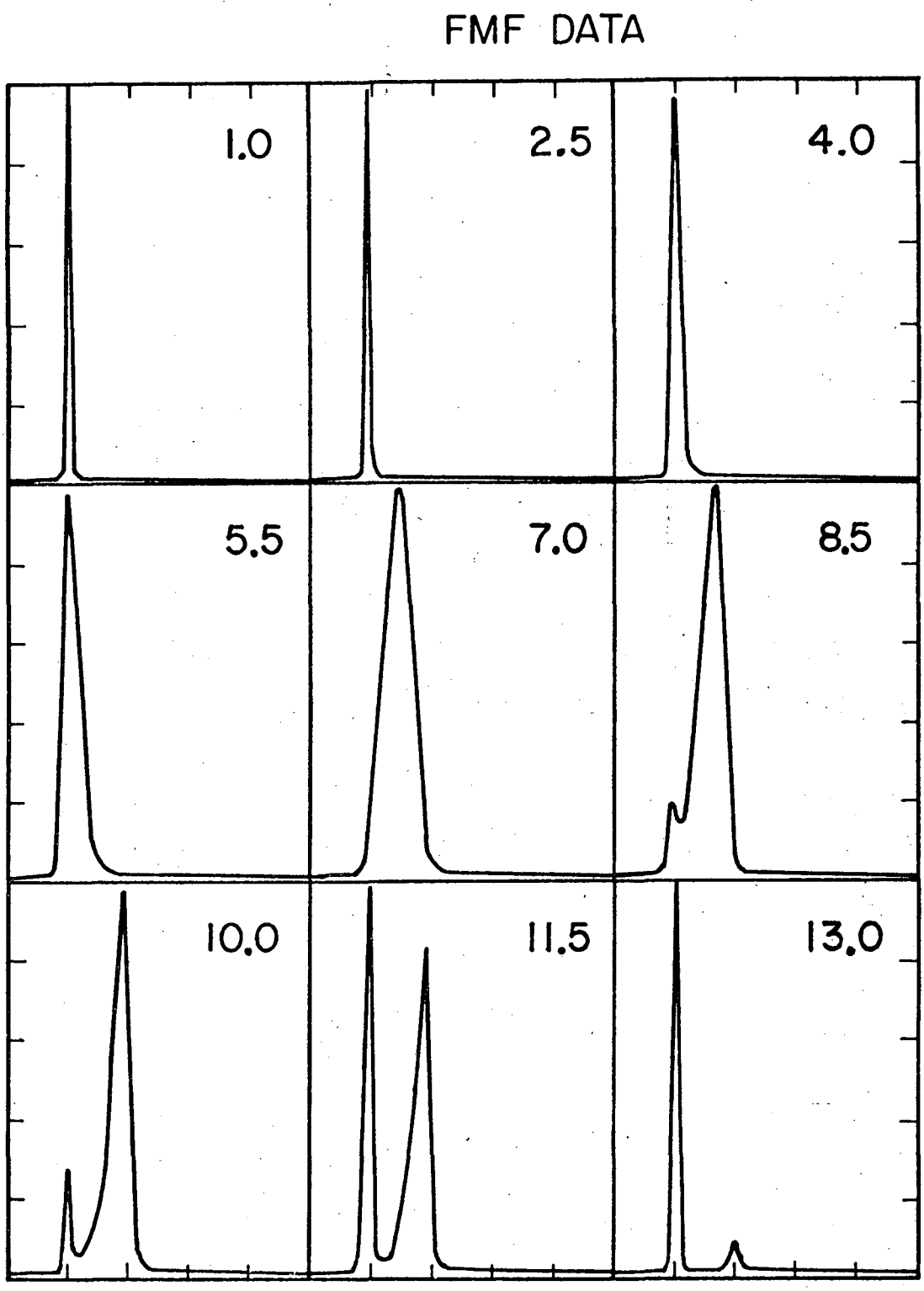

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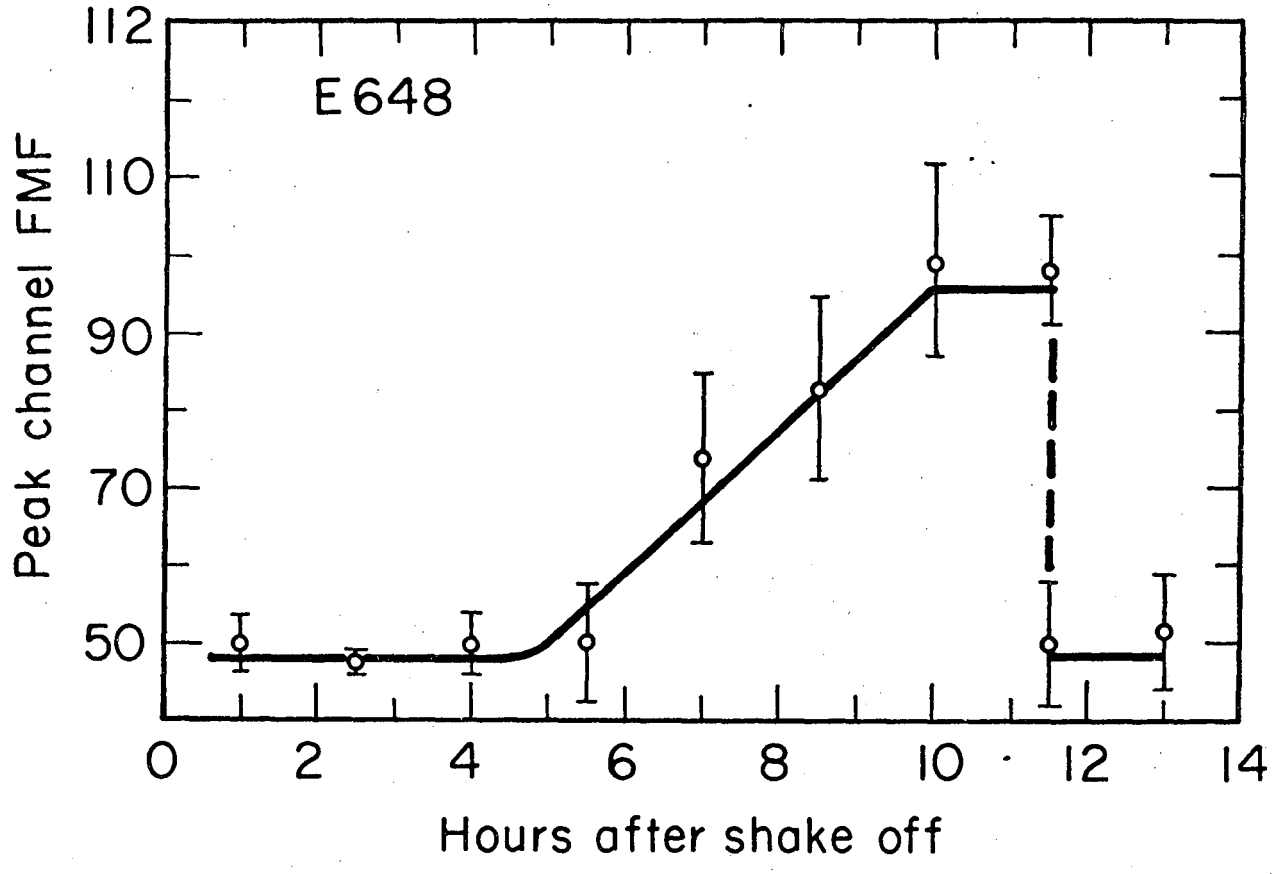

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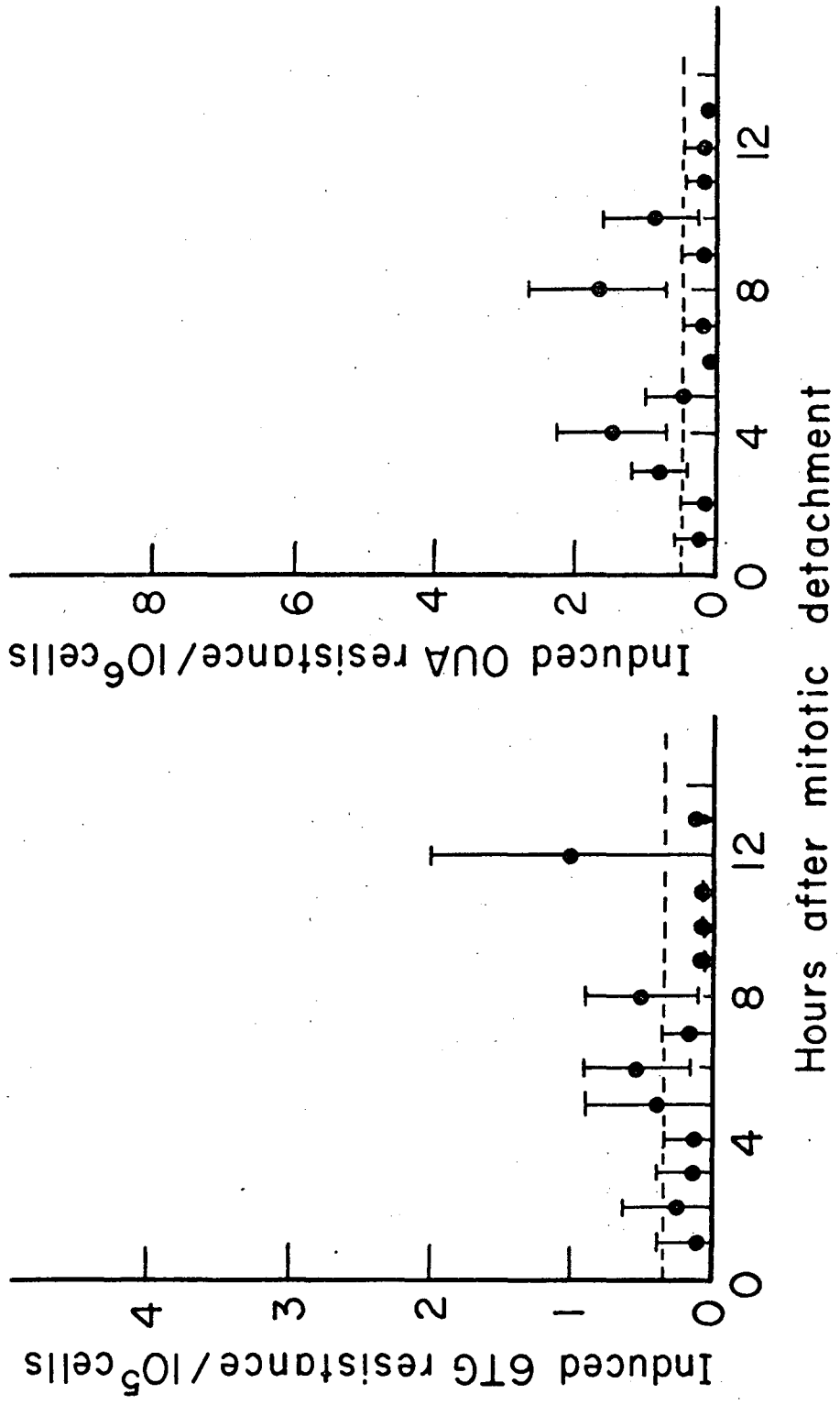

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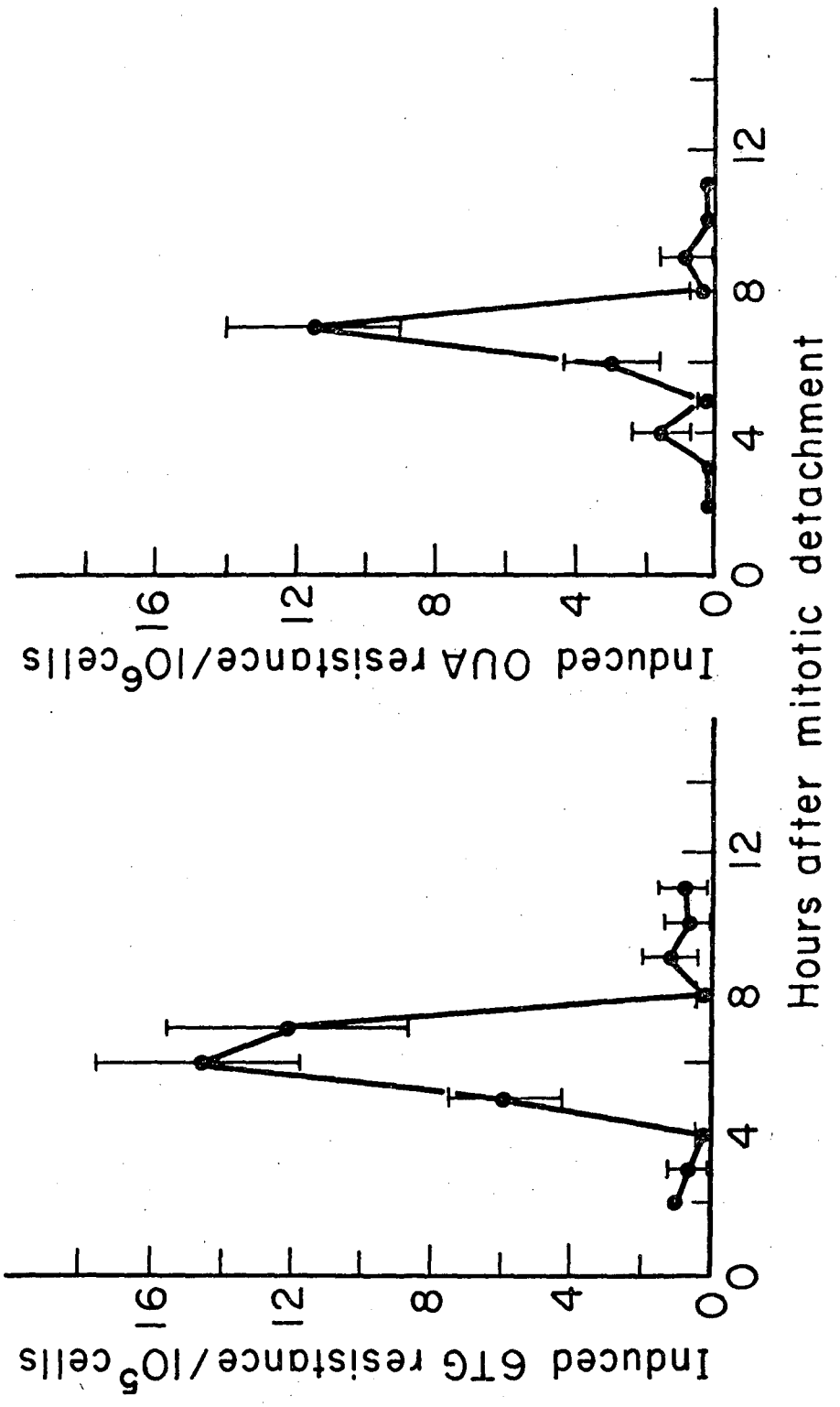

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Fig. 9

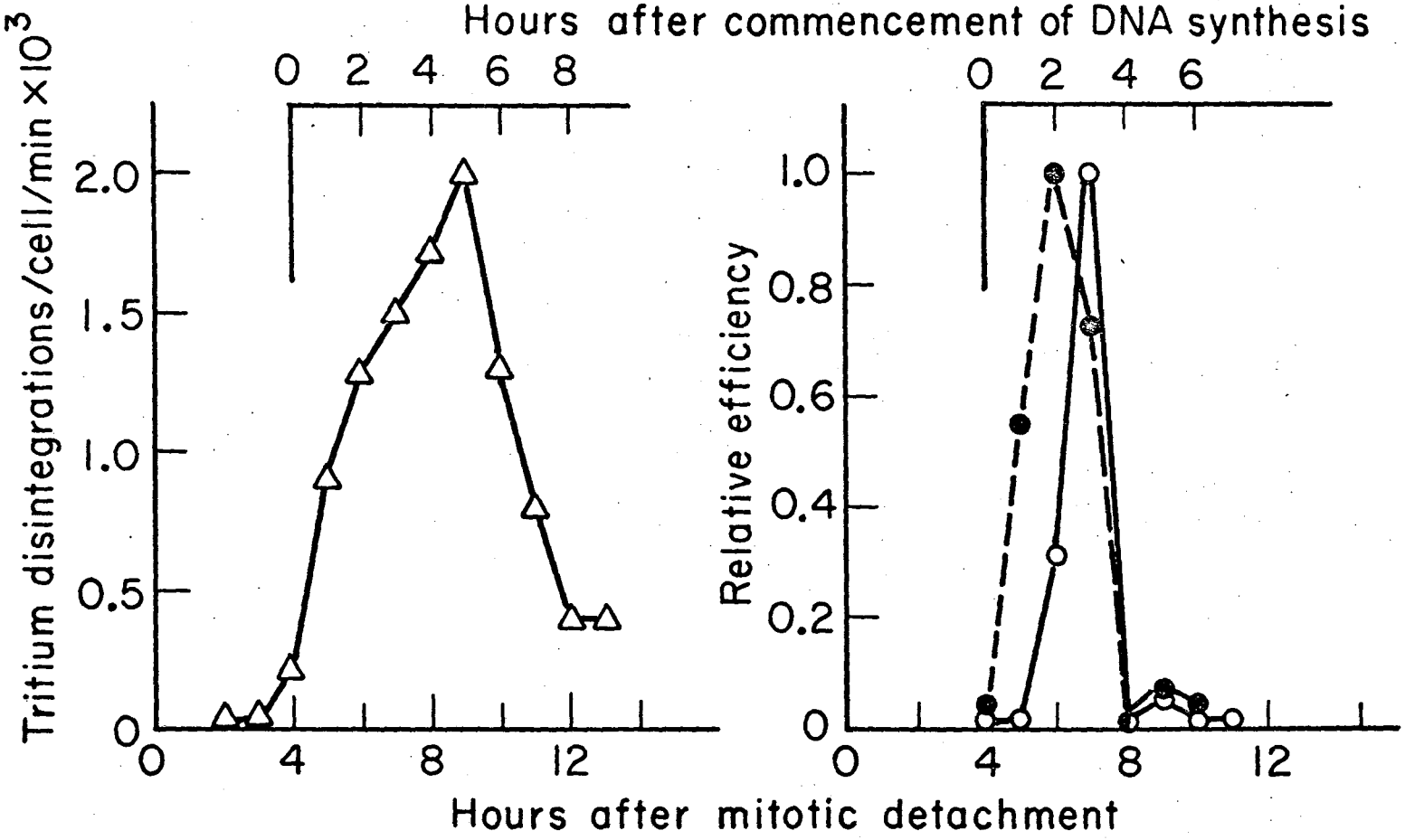

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Fig. 10

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