

THE THYMIDINE POOL IN GRASSHOPPER NEUROBLASTS DURING MITOSIS

WILLIAM M. LEACH. From the Institute of Radiation Biology, Department of Zoology and Entomology, The University of Tennessee, Knoxville, Tennessee. The author's present address is Radiation Bio-Effects Program, National Center for Radiological Health, United States Public Health Service, Rockville, Maryland 20852

In an earlier study (10), it was shown that exogenous thymidine is incorporated into a precursor "pool" during the period between deoxyribonucleic acid (DNA) syntheses. The possibility was not examined, however, that the ability of cells to incorporate thymidine into the pool varied with the mitotic stage at the time of exposure. Available evidence indicates that the ability of cells to incorporate thymidine into a precursor pool may vary with cell stage. First, in situ synthesis of

DNA precursors appears to be temporally related to DNA synthesis (9). Phosphorylation of thymidine occurs only near and during DNA synthesis in some systems (1, 8, 19). Finally, exogenous thymidine is retained in a DNA synthesis (18). The present study was undertaken so that it could be determined whether the relative amount of exogenous thymidine incorporated into a precursor pool of neuroblasts fluctuates during the period between DNA syntheses.

MATERIALS AND METHODS

Embryos of the grasshopper *Chortophaga viridifasciata* (De Geer) at an age equivalent to 14–15 days of development at 26°C were used for this study. Techniques of dissection and preparation of hanging-drop cultures have been described in detail (5). Criteria for determining cell stages have been previously reported (2, 6). Manipulations of embryos were performed at 26 ± 2°C.

Dissected embryos were placed in isotonic culture medium (16) which contained 10 μCi/ml (1.46 × 10⁻⁵ M) tritiated thymidine¹-(³HTdR) (specific activity 5.4 Ci/mmol) obtained from New England Nuclear Corp., Boston. During the 1st 10 min of exposure to ³HTdR, neuroblasts were mapped (4, 11) in desired stages of the cell cycle. Mapped neuroblasts were subsequently reidentified either in hanging-drop cultures or in stained sections. After exposure to ³HTdR, embryos were either fixed immediately or rinsed successively in two changes of culture medium containing 1.46 × 10⁻³ M (excess) unlabeled thymidine (Grade A, California Corp. for Biochemical Research), and in two changes of medium without thymidine. The sequence is hereinafter referred to as "rinse." It was completed in 15 min.

Tissue was fixed for 8 min in Newcomer's fluid (14), dehydrated, cleared, and embedded in paraffin. Sections 5 μ thick were mounted on slides, and radioautograms were prepared (12) and stained with 0.1% toluidine blue in 30% ethanol (15).

Silver grains in emulsion over mapped cells were counted within a circular area 30 μ² at the plane of the image. Grains can be counted more nearly accurately within this small area than within an entire nucleus (average cross-sectional area, 156 μ²). Grain numbers from counts of five randomly selected areas over each nucleus were averaged. The background level was determined for each slide by

¹ Samples of ³HTdR were tested for contamination by ascending paper chromatography with 86 *n*-butanol : 14 water. Chromatographs were scanned in either a chromatograph scanner or a gas flow counter. Lots of ³HTdR with more than 5% contamination were purified chromatographically.

counting grains in 25 randomly selected areas near, but not over, tissue. Background grain numbers averaged one to two grains per 113 μ².

RESULTS AND CONCLUSIONS

Onset of DNA Synthesis at 26°C

So that the time of onset of DNA synthesis in relation to the beginning of middle telophase at 26°C could be determined, neuroblasts were exposed to ³HTdR at the beginning of middle telophase, and fixed after various exposure times. At 26°C, incorporation of ³HTdR begins about 20 min after the cell enters middle telophase (see Fig. 1). Initial incorporation is rapid. The average duration of middle telophase at 26°C is 38 ± 3 min; therefore, DNA synthesis begins at approximately the middle of this stage, which affirms the conclusions of Gaulden (7) and McGrath (11). In succeeding experiments, neuroblasts were fixed 30 min after the beginning of middle telophase, or about 10 min after onset of DNA synthesis at 26°C.

Retention of ³HTdR in Stages of the Cell Cycle Not Associated with DNA Synthesis

As a test of the possibility that ³HTdR retained in neuroblasts varies with the mitotic stage, neuroblasts in middle prophase, prometaphase, metaphase, and anaphase were mapped in each hanging-drop culture during exposure to ³HTdR. The embryos were then removed from the cover glass, "rinsed," and again made into hanging-drop cultures, with medium lacking ³HTdR. The time that each cell entered middle telophase was recorded, and 30 min after the last mapped neuroblast entered middle telophase, the cells were fixed. Grain numbers from radioautograms of the cells (Fig. 2) were compared with the calculated regression line, $y = 1.02x - 8.7$, for the data in Fig. 1. The grain numbers of cells exposed during prometaphase and metaphase deviate more from the regression line than those of cells exposed during middle prophase and anaphase. It appears, therefore, that the prometaphase and metaphase neuroblasts incorporated less ³H per unit time after onset of DNA synthesis than those exposed during either middle prophase or anaphase.

So that the difference could be confirmed between middle prophase cells and prometaphase or metaphase cells with respect to incorporation

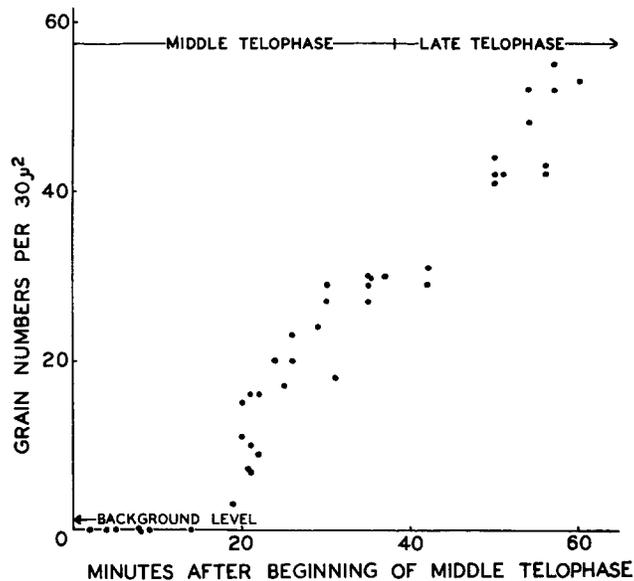


FIGURE 1 Onset of incorporation of $^3\text{HTdR}$ in neuroblasts in middle telophase. Cells were exposed to $^3\text{HTdR}$ between the beginning of middle telophase and fixation. Each point represents a single neuroblast. Emulsion exposure time, 5 days.

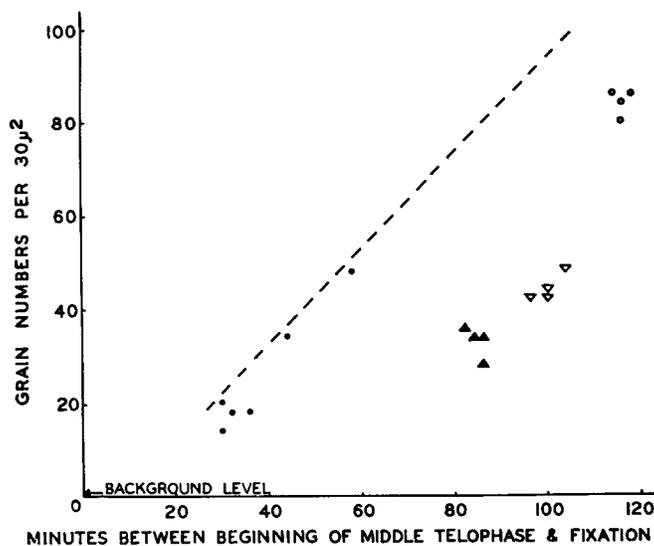


FIGURE 2 Incorporation of $^3\text{HTdR}$ in neuroblasts exposed during middle prophase, prometaphase, metaphase, or anaphase, and fixed after varying times in middle telophase. Dashed line represents regression, $y = 1.02x - 8.7$, calculated for data in Fig. 1. Neuroblasts exposed originally during middle prophase: ●; prometaphase, ▲; metaphase, ▽; anaphase, ○. Emulsion exposure time, 5 days. See text for additional details.

of $^3\text{HTdR}$, the preceding experiment was repeated with modifications. During exposure of each culture, cells were mapped in only one mitotic stage between early prophase and early telophase. From among the mapped cells, only those neuroblasts that entered middle telophase within 5 min of each other were selected for radioautographic analysis. (The time of entry into middle telophase was estimated for neuroblasts mapped during anaphase and early telophase). Grain numbers,

presented in Fig. 3, show that neuroblasts which were exposed to $^3\text{HTdR}$ during prometaphase and metaphase incorporated less $^3\text{HTdR}$ in the subsequent DNA synthesis period than did neuroblasts exposed during prophase or early telophase. Grain numbers from neuroblasts exposed during anaphase range between these two levels of incorporation. It can be concluded, therefore, that the retention of $^3\text{HTdR}$, or its derivatives other than DNA, is less during prometaphase and

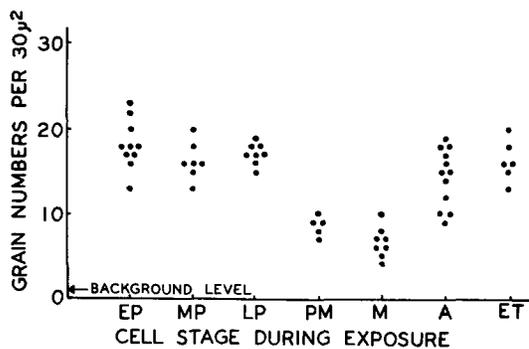


FIGURE 3 Incorporation of $^3\text{HTdR}$ in middle telophase after exposure during individual stages. Abscissa shows stage during exposure to $^3\text{HTdR}$. Neuroblasts were fixed approximately 30 min after beginning of middle telophase. Symbols: *EP* = early prophase, *MP* = middle prophase, *LP* = late prophase, *PM* = prometaphase, *M* = metaphase, *A* = anaphase, *ET* = early telophase. Emulsion exposure time, 5 days.

metaphase than during other cell stages in the portion of the cell cycle of no DNA synthesis.

Effect of Excess Unlabeled Thymidine on the Availability of $^3\text{HTdR}$

So that it could be determined whether the "rinse" with excess thymidine was effective in reducing $^3\text{HTdR}$ in the precursor pool, neuroblasts in metaphase were mapped during a 10-min exposure to $^3\text{HTdR}$. Embryos were then treated with excess thymidine for 5, 10, or 20 min. After an additional rinse (5 min) in medium without thymidine, mapped cells were relocated in cultures, and fixed 30 min after the beginning of middle telophase (time of beginning of middle telophase was estimated for cells in excess thymidine for 20 min). The distribution of grain numbers over neuroblasts reidentified in radioautograms (Fig. 4) is similar. Under the conditions employed, therefore, excess thymidine is ineffective in diluting intracellular $^3\text{HTdR}$ from neuroblasts.

DISCUSSION

In terms of DNA synthesis, the neuroblast cell cycle may be considered to consist of two parts, a synthetic period and a nonsynthetic period. Equivalent amounts of $^3\text{HTdR}$ are retained when exposure occurs in the portions of the nonsynthetic period just after completion of one synthesis period and just before onset of another synthesis

period. Less $^3\text{HTdR}$ is retained in the mid-portion of the nonsynthetic period which corresponds to prometaphase and metaphase when the nuclear boundary is lacking. (The electron microscope studies of neuroblasts (17) reveal that membranes begin to form around individual chromosomes during anaphase). Thus retention of $^3\text{HTdR}$, or its derivatives other than DNA, in the neuroblast may be related to the organization of the nucleus. Support for this idea comes from observations that the soluble derivatives of $^3\text{HTdR}$ in *Tetrahymena* are localized in nuclei (13). It is possible that a $^3\text{HTdR}$ derivative may be formed in the nucleus more efficiently when the nucleus is intact than when the nuclear boundary is lacking.

One other possible explanation is that permeability of the cell membrane may change in neuroblasts during mid-mitosis. Carlson (3) has reported decreased viscosity in neuroblasts in mid-mitosis. In neither Carlson's nor the present study is it possible, however, to distinguish between changed permeability and cellular reorganization. The viscosity change might be a result of increased permeability to water; the mid-mitotic reduction in the amount of retained $^3\text{HTdR}$, as well as failure to detect dilution of retained $^3\text{HTdR}$, may be the results of decreased membrane permeability to thymidine. It should be pointed out, therefore, that failure to detect dilution is not conclusive evidence for no dilution.

SUMMARY

Neuroblasts of the grasshopper *Chortophaga viridifasciata* (De Geer) were exposed to tritiated thy-

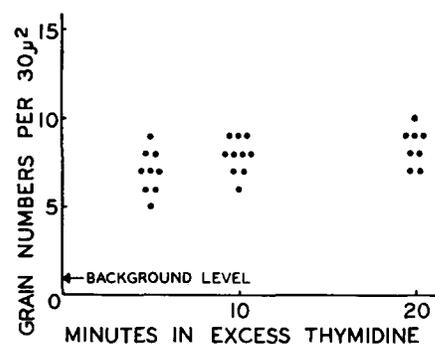


FIGURE 4 Incorporation of $^3\text{HTdR}$ in neuroblasts after incubation in excess unlabeled thymidine. Cells were exposed to $^3\text{HTdR}$ during metaphase and fixed about 30 min after the beginning of middle telophase. Emulsion exposure time, 5 days.

midine (³HTdR) during several stages of the cell cycle between early prophase and early telophase, rinsed with unlabeled thymidine 100 times more concentrated than the ³HTdR, and fixed after the cells entered DNA synthesis in middle telophase. Two main points result from radioautographic analysis of the cells: (1) Neuroblasts exposed during prometaphase and metaphase incorporate less ³HTdR than those exposed

during early, middle, or late prophase, or early telophase; and (2) dilution of ³HTdR by unlabeled thymidine was not detected in neuroblasts exposed during metaphase.

This study was supported in part by the United States Atomic Energy Commission under Contract No. AT-(40-1)-2575.

Received for publication 7 July 1967, and in revised form 5 September 1967.

REFERENCES

1. BOLLUM, F. J., and V. R. POTTER. 1959. Nucleic acid metabolism in regenerating rat liver. VI. Soluble enzymes which convert thymidine to thymidine phosphates and DNA. *Cancer Res.* **19**:591.
2. CARLSON, J. G. 1941. Effects of X-radiation on grasshopper chromosomes. *Cold Spring Harbor Symp. Quant. Biol.* **9**:104.
3. CARLSON, J. G. 1946. Protoplasmic viscosity changes in different regions of the grasshopper neuroblast during mitosis. *Biol. Bull.* **90**:109.
4. CARLSON, J. G. 1961. The grasshopper neuroblast culture technique and its value in radiobiological studies. *Ann. N.Y. Acad. Sci.* **95**:932.
5. CARLSON, J. G., and M. E. GAULDEN. 1964. Grasshopper neuroblast techniques. In *Methods in Cell Physiology*. D. M. Prescott, editor. Academic Press Inc., New York. **1**:229.
6. CARLSON, J. G., and A. HOLLAENDER. 1948. Mitotic effects of ultraviolet radiation of the 2250 Å region, with special reference to the spindle and cleavage. *J. Cellular Comp. Physiol.* **31**:149.
7. GAULDEN, M. E. 1956. DNA synthesis and X-ray effects at different mitotic stages in grasshopper neuroblasts. *Genetics.* **41**:645.
8. HOTTA, Y., and H. STERN. 1963. Molecular facets of mitotic regulation. I. Synthesis of thymidine kinase. *Proc. Natl. Acad. Sci. U. S.* **49**:648.
9. LARK, K. G. 1963. Cellular control of DNA biosynthesis. In *Molecular Genetics*. J. H. Taylor, editor. Academic Press Inc., New York. **1**:153.
10. LEACH, W. M. 1964. Retention of tritiated thymidine in grasshopper neuroblasts. *Exptl. Cell Res.* **35**:201.
11. McGRATH, R. A. 1963. X-ray-induced incorporation of tritiated thymidine into deoxyribonucleic acid of grasshopper neuroblast chromosomes. *Radiation Res.* **19**:526.
12. McGRATH, R. A., W. M. LEACH, and J. G. CARLSON. 1963. A squash technique for autoradiography of grasshopper neuroblasts. *J. Roy. Microscop. Soc.* **82**:55.
13. MILLER, O. L., JR., G. E. STONE, and D. M. PRESCOTT. 1964. Autoradiography of soluble materials. *J. Cell Biol.* **23**:654.
14. NEWCOMER, E. H. 1953. A new cytological and histological fixing fluid. *Science.* **118**:161.
15. PEARSE, A. G. E., editor. 1962. *Histochemistry, Theoretical and Applied*. Little, Brown and Company, Boston. 2nd edition. 998.
16. SHAW, E. I. 1956. A glutamic acid-glycine medium for prolonged maintenance of high mitotic activity in grasshopper neuroblasts. *Exptl. Cell Res.* **11**:580.
17. STEVENS, B. J. 1965. The fine structure of the nucleolus during mitosis in the grasshopper neuroblast cell. *J. Cell Biol.* **24**:349.
18. STONE, G. E., O. L. MILLER, JR., and D. M. PRESCOTT. 1965. H³-thymidine derivative pools in relation to macromolecular DNA synthesis in *Tetrahymena pyriformis*. *J. Cell Biol.* **25**:171.
19. WEISSMAN, S. M., R. M. S. SMELLIE, and J. PAUL. 1960. Studies on the biosynthesis of deoxyribonucleic acid by extracts of mammalian cells. IV. The phosphorylation of thymidine. *Biochim. Biophys. Acta.* **45**:101.