AUTORADIOGRAPHY WITH MEIOTIC CHROMOSOMES OF THE MALE NEWT (*TRITURUS VIRIDESCENS*) USING H^3 -THYMIDINE¹

DONALD E. WIMBER² AND WOLF PRENSKY³

Biology Department, Brookhaven National Laboratory, Upton, N.Y.

Received August 14, 1963

THE very specialized meiotic events that occur in all sexually reproducing organisms have not been investigated in any great detail by means of high resolution tritium autoradiography. Most of the work so far has been done with animals because of technical difficulties in obtaining uptake of labelled nucleosides in the microsporocytes of plants (TAKATS 1962). LIMA-DE-FARIA (1959, 1961, 1962) has studied the incorporation of tritiated thymidine by spermatocytes of the grasshopper and by oocytes of the Dipteran, *Tipula*. His studies demonstrated a marked asynchrony of deoxyribonucleic acid replication during the premeiotic DNA synthetic period. These observations were carried to the prophase of meiosis. MONESI (1962) studied the duration of the DNA synthetic period (S phase) prior to meiosis in the male mouse and concluded that there is a gradual lengthening of the S phase during successive spermatogonial divisions, culminating with the last premeiotic DNA replication lasting approximately 14 hours. TAYLOR (1958, 1959) utilized P^{32} and C^{14} -labelled glycine and orotic acid in autoradiographic investigations of *Lilium* and *Tulbaghia* microsporocytes. He concluded that DNA was synthesized prior to leptotema.

This paper reports an autoradiographic study of the meiotic chromosomes of the male newt labelled with H^3 -thymidine. It will be shown that aside from the main period of DNA synthesis in pre-prophase spermatocytes, a slow DNA synthesis occurs during a part of meiotic prophase.

MATERIALS AND METHODS

The adult male newt (*Triturus viridescens*) was the experimental subject. The newts were collected in North Carolina during February and were maintained at room temperature (20–22°C) with biweekly feedings for two months before the start of the experiment. The newt is meiotically inactive during the winter months; by April meiotic phases are frequent in the anterior portion of the testes. Newts weighing between 4.25 and 4.75g were injected intraperitoneally with 10 μ c H^3 -thymidine (3.0 c/mm, New England Nuclear, in 0.01 ml distilled water). After injection the testes were removed, fixed in acetic alcohol (1:3), and

¹ Research carried out at Brookhaven National Laboratory under the auspices of the U. S. Atomic Energy Commission.

² Present address: Biology Department, University of Oregon, Eugene, Oregon.

³ Supported by Public Health Service Postdoctoral Research Fellowship GM 12,602.

stained by the Feulgen method, and bits of the anterior portion of the testes were squashed on slides. Autoradiographs were prepared using Kodak NTB liquid emulsion and stored at -20°C for approximately eight months. They were developed using standard procedures.

Reduced silver grains over metaphase I figures were counted microscopically where possible, to test for the rate of DNA synthesis. In cases where the number of grains was so excessive as to be uncountable, an arbitrary number of 180 was assigned to the cell. Background grain counts were in general less than two grains per metaphase I nucleus. Grain counts per cell on unlabelled material were usually characterized by a Poisson distribution. Labelled cells not only had a higher grain count, but the distribution, for a given slide, approached a normal curve. All grain counts were corrected by subtracting five grains per cell, a value which is well above background. Counts were taken over at least 200 metaphase I figures for each daily interval. In slides from some of the newts, the number of grains over metaphase I figures ranges between 40 and 170. In such material it was possible to test for synchrony of DNA replication between homologous chromosomes. The grain counts were taken on each of a pair of synapsed homologues. For this part of the study only those cells were used in which four or more of the chromosome pairs were well spread.

A test of the time of availability of H^3 -thymidine in the newt was made by injecting six newts with $20\text{ }\mu\text{c}$ H^3 -thymidine each, and killing them at 40 min, 4 hr and 6 hr later. Autoradiographs of squashed testes were prepared and exposed long enough to give an average of about 15 grains per labelled cell. Grains were counted over at least 80 labelled cells from each newt.

RESULTS

Figure 1 shows the data from autoradiographic grain counts over metaphase I (M I) figures plotted with respect to the time interval between injection of H^3 -thymidine and fixation. There is a small amount of label seen over M I chromosomes between three to five days after labelling. At least three-fourths of the M I chromosome sets at these time periods showed grain counts which were above background values. There is a large amount of label seen over early prophase stages (around leptonema) concomitantly with the light label over M I chromosomes. Considering the fact that it takes at least 11 days for those heavily labelled nuclei to progress from early prophase to M I (distance on abscissa from Day 3 to Day 13), we feel that the lightly labelled M I cells at Days 3 to 5 were in zygonema-pachynema at the time the label was available. A direct examination of zygonema-pachynema, in squashes prepared from newts killed a few hours after injection, revealed a small amount of label over these stages. The estimation of the amount of label incorporated during prophase is at present very inaccurate. The exposure times were such that during the middle of the main DNA synthetic period the autoradiographs were saturated (we used an arbitrary value of 180). The effect of the overexposure of cells rapidly synthesizing DNA was to magnify the size of the fraction synthesized in prophase, whereas the rigid correction for

FIGURE 1.—The number of grains over metaphase I chromosomes plotted against time after injection with H^3 -thymidine. A small amount of label is seen over M I chromosomes between 3–5 days after injection (labelled during prophase) as well as a large amount of label between 14–26 days (labelled during premeiotic DNA synthesis phase). 180 grains was taken as an arbitrary upper limit, and indicates total or partial saturation of the autoradiograph.

background tended to minimize it. A reasonable estimate, based on areas under the histogram in Figure 1, is that about 2.0 percent, or less, of the genome is synthesized during prophase.

From the sixth to the 12th day following injection, there is a period during which little or no label is detected over M I chromosomes. Beginning at the 13th day, an increasing amount of label is seen over the M I figures. Saturation of the autoradiograph over M I chromosomes is reached at Day 17 and lasts until Day 24. On the 25th and 26th days there is a decrease in the number of grains over the M I figures. Although our material does not extend past the 26th day, a rough estimate of the duration of the main DNA synthetic period prior to meiosis can be made. We estimate this to be approximately 13 days (distance on the abscissa between the 14th and 26th days). Similarly, prophase of meiosis lasts about 12 days.

Our estimate of the duration of the DNA synthetic period is based on the assumption that the labelling from a given H^3 -thymidine injection is complete within one day. To test this hypothesis we injected newts with H^3 -thymidine and killed them at 40 min, 4 hr and 6 hr after injection. The average numbers of silver grains seen over labelled nuclei at various times after injection are given in Table 1. The data indicate that there is a small but insignificant rise in the amount of label seen over nuclei between the 40 minute fixation and the 4 hour fixation; there is no increase in label between 4 and 6 hours. The greatest amount of tritium has been incorporated within the first 40 minutes, and we conclude that the thymidine is available for less than 4 hours.

The asynchrony of DNA replication in the whole genome is a very striking phenomenon in this material (Figures 2 and 3). In general, nonrandom labelling

TABLE 1

Availability time of H³-thymidine for incorporation by spermatocytes of Triturus viridescens

Source	Treatment Means (grains/cell)* Fixation time after injection			F
	40 min	4 hr	6 hr	
	12.0	14.9	15.2	
Analysis of Variance†				
	Degress of freedom	Mean square		
Between treatments	2	600.25	594.31**	
a) between 40 min and later	1	1,199.90	1,188.01**	
b) between 4 and 6 hr	1	.60	<1.00	
Within treatments (Error)	3	1.01	.....	

* Two animals per treatment.

† SNEDECOR 1956.

** Significant at the 1 percent level (tabled values of *F*, 30.82 for 2 *d.f.* and 34.12 for 1 *d.f.*).

could be seen over most labelled cells except in cases where there was total saturation of the autoradiograph over figures at diakinesis and M I.

The number of grains over homologous chromosome pairs was counted at M I from newts killed at 16, 25 and 26 days after injection, to test for unequal rates of DNA synthesis in homologues. By our previous definition, these cells were in the beginning or end of the main S phase when the H³-thymidine was available. 789 pairs were counted from 126 cells. Not all of the chromosome pairs in each cell were counted. The number of grains over at least four pairs of homologues from each cell was tested for significant differences from equality (assuming a random sample of homologues from each cell). The percentage of the chromosome pairs with significant chi-square differences at the 1, 5, 10, 25 and 50 percent levels are presented in Table 2. In general, the data agree with the expected percentages assuming equal replication rates in homologous chromosomes.

The number of grains over the chromosomes reflects the amount of H³-thymidine taken up into the DNA and this is usually equated to the rate of DNA syn-

TABLE 2

Percentage of chromosome pairs having significantly different grain numbers between homologues

Animal	Slide	Number of chromosome pairs	Percent homologous chromosome pairs differing significantly from randomness at the stated probability levels				
			.01	.05	.10	.25	.50
1	16A	291	1.37	5.84	11.00	24.05	51.89
	16B	76	1.32	5.26	11.84	36.84*	51.32
	16C	42	0.00	2.38	9.52	16.67	42.86
	16F	67	1.49	5.97	10.45	19.40	44.78
2	23F	27	3.70	7.41	11.11	22.22	62.96
	23B	70	0.00	4.29	8.57	27.14	45.71
	23A	102	0.98	7.84	17.65**	38.24**	58.82
3	24A	109	0.92	8.26	15.60	33.94*	57.80

*, ** Goodness-of-fit test significant at the .05 and .01 levels, respectively.

FIGURES 2-5.—Autoradiographs of meiotic phases from the testes of the male newt. H^3 -thymidine was injected more than two weeks prior to fixation. The chromosomes were labelled during the last DNA synthetic period before meiosis. FIGURE 2.—Pachynema showing very localized labelling at certain sites on the chromosomes. The elongated structure overlying the nucleus is a sperm. FIGURE 3.—Anaphase I. The chromatids are visible, being held together at the centromere. FIGURES 4 and 5.—Metaphase I. The chromosome pairs indicated by the arrows show inequalities in the labelling over the homologues.

thesis. Hence, the rate of DNA synthesis in homologous chromosomes at the time the label was available was approximately equal. However, the sites of synthesis on the chromosome arms, as indicated by clusters of grains, were not always found at similar locations. Figures 4 and 5 show examples of what appear to be unequal distributions of grains over homologous portions of paired chromosomes. Some of these unequal distributions could possibly be caused by subtle differences in the distance of the emulsion from different parts of the chromosome; we cannot be sure of this and hence, we are only presenting these as tentative results until more extensive observations can be made.

DISCUSSION

It has been claimed for several years that there is no DNA synthesis during meiotic prophase. TAYLOR (1958, 1959), in particular, has studied meiosis in a few plants and has found no indication of DNA synthesis during prophase. On the other hand, HOTTA and STERN (1961) studied thymidine phosphorylation in suspensions of *Lilium* and *Trillium* microsporocytes at various times of development and found a small rise in phosphorylation during pachynema. They, however, attach no significance to this finding since they fail to mention it in the text of their paper. PRENSKY (1962) detected tritium incorporation autoradiographically from H^3 -thymine in pachynema of *Lilium*, but this observation could not be readily repeated. Our finding of a small but consistent amount of DNA synthesis during pachynema in the newt leads one to a reconsideration of some of the older theories of crossing over.

The synthesis that occurs during prophase could presumably result from either a reunion of broken chromatids at crossing over or from continued synthesis in chromosomes that had not finished replicating by the beginning of prophase. Our data suggest that the DNA synthesis in prophase is a discrete event rather than a continuation of unfinished synthesis, but we cannot be sure of this. Our preliminary data on the spermatogonial stages in the newt indicate that the duration of the S phase is 24 hours or less, while we have shown that the S period prior to meiosis is about 13 days. MONESI (1962) showed that the last two S phases in the male mouse before meiosis were about twice as long as some of the earlier periods. Considering such a slow DNA synthesis rate in our material, it is quite possible that it could continue on into prophase partially undetected and then end with a surge. It is of interest to recall BELLING's hypothesis of crossing over at this point. BELLING (1933) thought that chromosomes in zygonema paired prior to duplication and that crossing over resulted from an exchange between chromatids during their synthesis. This is an attractive theory to apply to this case, although in a somewhat modified form. Intimately paired homologues could finish DNA replication in prophase, even though most of the DNA synthesis for the meiotic division occurs during interphase. Crossing over could result from errors during this final DNA synthesis while the homologues are paired. Our finding seems to invalidate TAYLOR's (1957) criticism that BELLING's hypothesis of crossing over is untenable because DNA synthesis is restricted to interphase.

Although a BELLING-type interpretation of crossing over is possible, we cannot rule out the possibility that the DNA synthesis seen during prophase results from actual chromatid breakage and repair. The very much elongated prophase, together with a different coiling cycle from mitosis, could initiate breaks leading to crossing over (cf. DARLINGTON 1937). The data of TAYLOR, HAUT, and TUNG (1962) indicate that healing enzymes may be present after the main DNA synthetic period has ceased. He obtained restitution of potential breaks induced by fluorodeoxyuridine in root tip cells by adding exogenous thymidine while the cells were either in the post-DNA synthetic period or in prophase. Potentially, breaks induced by coiling, or skips in the base sequences, or both, could be repaired during meiotic prophase if such healing enzymes were present.

LIMA-DE-FARIA (1959, 1961) studied the asynchrony of DNA replication during the DNA synthetic phase prior to meiosis in the grasshopper. He found that the rate of DNA synthesis was far from constant. In the newt we find an alteration in the rate of DNA synthesis similar to that found in the grasshopper. Analogous changes in rate of synthesis have been observed in mitotic cells from both plants and animals (cf. HOWARD and DEWEY 1961; WIMBER 1961; TAYLOR 1960; PAINTER 1961; DONNELLY and NEWCOMER 1963; GILBERT, MULDAL, LAJTHA, and ROWLEY 1962). LIMA-DE-FARIA (1961) also found that the large heterochromatic blocks in the chromosomes of the grasshopper were partially synthesized at a later time than euchromatin. The newt also has a large amount of heterochromatin. However, the heterochromatic areas do not seem to be specifically duplicated during the last part of DNA synthesis.

Even though the rate of DNA synthesis on homologues was approximately equal at any one time, there did seem to be an occasional difference between the sites of replication. The amount of these differences is very difficult to quantitate. We did see enough of these inequalities to convince us that this was probably a real phenomenon in some chromosomes. Unfortunately our preparations did not allow us to examine a known pair of chromosomes in several different cells at different times after H^3 -thymidine treatment. Further studies with improved techniques may allow us to do this in the future.

SUMMARY

The nuclei in the last DNA synthetic period prior to meiosis were labelled by injecting male newts with $10 \mu c$ H^3 -thymidine. The newts were killed at daily intervals and autoradiographs of testicular squashes were prepared. A slow DNA synthesis was detected during part of meiotic prophase (pachynema) which may be related to genetic crossing over. The period between the termination of the main DNA synthetic period during interphase and metaphase I in meiosis is about 12 days. The DNA synthetic period prior to meiosis is about 13 days long. The rate of DNA synthesis during the premeiotic synthetic period varies within a wide range. The rates of DNA synthesis on homologous chromosomes were equal at any one time. Tentative observations indicate that the sites of DNA synthesis may not always be at the same location.

ACKNOWLEDGMENT

We wish to thank DR. KEITH THOMPSON for aid with the statistical computations

LITERATURE CITED

- BELLING, J., 1933 Crossing-over and gene rearrangement in flowering plants. *Genetics* **18**: 388-413.
- DARLINGTON, C. D., 1937 *Recent Advances in Cytology*. Second edition. Blakiston, Philadelphia.
- DONNELLY, G. M., and E. H. NEWCOMER, 1963 Autoradiographic patterns in cultured leukocytes of the domestic fowl. *Exptl. Cell Res.* **30**: 363-368.
- GILBERT, C. W., S. MULDAL, L. G. LAJTHA, and J. ROWLEY, 1962 Time-sequence of human chromosome duplication. *Nature* **195**: 869-873.
- HOTTA, Y., and H. STERN, 1961 Transient phosphorylation of deoxyribosides and regulation of deoxyribonucleic acid synthesis. *J. Biophys. Biochem. Cytol.* **11**: 311-319.
- HOWARD, A., and D. L. DEWEY, 1961 Nonuniformity of labeling rate during DNA synthesis. *Exptl. Cell Res.* **24**: 623-624.
- LIMA-DE-FARIA, A., 1959 Differential uptake of tritiated thymidine into hetero- and euchromatin in *Melanoplus* and *Secale*. *J. Biophys. Biochem. Cytol.* **6**: 457-466.
- 1961 Initiation of DNA synthesis at specific segments in the meiotic chromosomes of *Melanoplus*. *Hereditas* **47**: 674-694.
- 1962 Metabolic DNA in *Tipula oleracea*. *Chromosoma* **13**: 47-59.
- MONESI, V., 1962 Autoradiographic study of DNA synthesis and the cell cycle in spermatogonia and spermatocytes of mouse testis using tritiated thymidine. *J. Cell Biol.* **14**: 1-18.
- PAINTER, R. B., 1961 Asynchronous replication of HeLa S3 chromosomal deoxyribonucleic acid. *J. Biophys. Biochem. Cytol.* **11**: 485-488.
- PRENSKY, W., 1962 Uptake of thymine-methyl- H^3 by pachytene chromosomes of *Lilium longiflorum*. *Genetics* **47**: 977.
- SNEDECOR, G. W., 1956 *Statistical Methods*. Fifth edition. Iowa State College Press, Ames, Iowa.
- TAKATS, S. T., 1962 An attempt to detect utilization of DNA breakdown products from the tapetum for DNA synthesis in the microspores of *Lilium longiflorum*. *Am. J. Botany* **49**: 748-758.
- TAYLOR, J. H., 1957 The time and mode of duplication of chromosomes. *Am. Naturalist* **91**: 209-221.
- 1958 Incorporation of phosphorus-32 into nucleic acids and proteins during microgametogenesis of *Tulbaghia*. *Am. J. Botany* **45**: 123-130.
- 1959 Autoradiographic studies of nucleic acids and proteins during meiosis in *Lilium longiflorum*. *Am. J. Botany* **46**: 477-484.
- 1960 Asynchronous duplication of chromosomes in cultured cells of the Chinese hamster. *J. Biophys. Biochem. Cytol.* **7**: 455-463.
- TAYLOR, J. H., W. F. HAUT, and J. TUNG, 1962 Effects of fluorodeoxyuridine on DNA replication, chromosome breakage and reunion. *Proc. Natl. Acad. Sci. U. S. A.* **48**: 190-198.
- WIMBER, D. E., 1961 Asynchronous replication of deoxyribonucleic acid in root tip chromosomes of *Tradescantia paludosa*. *Exptl. Cell Res.* **23**: 402-407.