MEIOTIC DNA SYNTHESIS DURING MOUSE SPERMATOGENESIS

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ABSTRACT

The incorporation of radioactivity into various cells in the sequence of spermatogenesis was measured by preparing highly purified spermatozoan nuclei from the cauda epididymidis of mice at daily intervals after injection of [^{3}H]thymidine. The stages of differentiation of these sperm at the time of thymidine administration were calculated from the kinetics of spermatogenesis. The procedure for purification of sperm nuclei included sonication, mechanical shearing, and treatment with trypsin, DNase, Triton X-100, 2 M NaCl, and sodium dodecyl sulfate. DNA was isolated from these nuclei by treatment with dithiothreitol and pronase, followed by phenol extraction and ethanol precipitation.

The levels of radioactivity in the epididymal sperm head preparations were low (<13 dpm/mouse) for 27 days after injection, and then rose dramatically to over 4×10^4 dpm/mouse. Further experiments demonstrated that the 11 dpm of ^{3}H radioactivity contained in sperm heads at 21 or 26 days after injection of [^{3}H]TdR was significantly above background and contamination levels from other cells or other sources. Most of the radioactivity was in the sperm DNA and represented incorporation of tritium from [^{3}H]TdR into the nuclear DNA of meiotic cells at 0.002% of the rate of incorporation into S-phase cells. Little, if any, [^{3}H]TdR was incorporated into the DNA of spermatids. The levels of DNA synthesis during the meiotic prophase in the mouse appear to be much lower than those reported for other organisms.

A small amount of DNA synthesis is expected to occur during the meiotic prophase as a result of the processes of genetic recombination (Meselson, 1964; Whitehouse, 1963) and gene conversion (Holliday, 1964) which occur at this time. Although nearly all DNA synthesis ceases at the end of the premeiotic interphase (Taylor and McMaster, 1954; Monesi, 1962), it has been conclusively shown that a small amount of DNA corresponding to 0.4% of the genome is synthesized during the zygotene and pachytene stages of meiosis in the lily (Hotta, Ito and Stern, 1966; Hotta and Stern, 1971).

Any method of studying meiotic DNA synthesis must, therefore, be able to detect low levels of incorporation of labeled precursors and eliminate contamination from the active S-phase cells. With autoradiographic methods, S-phase cells can be identified and excluded from the counts, but the sensitivity is low. Liquid scintillation counting provides higher sensitivity but the separation of meiotic cells must be achieved.

Despite recent development of methods for separating testis cells and nuclei (Meistrich and Eng, 1972; Meistrich et al., 1973) only spermatozoan nuclei can be prepared in sufficient purity for this study. However, since the kinetics of spermatogenesis and epididymal sperm transport are synchronous (Oakberg, 1957; Orgebin-Crist, 1965), stable incorporation of tritiated thymidine ([^{3}H]TdR) into any spermatogenic cell type can be measured as radioactivity in sperm nuclei of mice sacrificed at a particular time after injection of [^{3}H]TdR. Using this approach, we have obtained data which indicate that meiotic DNA synthesis occurs in the male mouse at extremely low levels.

MATERIALS AND METHODS

Mice

Inbred C3Hf/Bu male mice between 9 and 15 wk of age with an average body weight of 30 g were used. They were maintained in a specific pathogen-free colony with a daily cycle of 12 h of light and 12 h of darkness.

Materials

Tritiated thymidine ([*methyl*-^{3}H]TdR, sp act between 17 and 23 Ci/mM), tritiated water (THO), and [^{3}H]arginine (L-arginine, 10 Ci/mM) for injection of mice, and [^{3}H]TdR (sp act 1.9 Ci/mM) for labeling Chinese hamster ovary (CHO) cells were obtained from Schwarz/Mann (Div. of Becton, Dickinson & Co., Orangeburg, N.Y.), kept under sterile conditions, and used within 3 mo of receipt. Bovine serum albumin (BSA, fraction V), Triton X-100, and crude DNase (beef pancreatic) were obtained from Sigma Chemical Company, St. Louis, Mo. Purified DNase (code:DPFF) was obtained from Worthington Biochemical Company, Freehold, N.J. Trypsin was obtained as a 2.5% solution of crude enzyme in saline (Grand Island Biological Co., Grand Island, N.Y.). Pronase (B grade, Calbiochem, San Diego, Calif.) was preincubated to eliminate nuclease activity. Sodium dodecyl sulfate (SDS) was purchased from Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N.Y., and NCS solubilizer from Amersham/Searle Corp., Arlington Heights, Ill. Most solutions used were sterilized by autoclaving or by Millipore filtration to minimize growth of micro-organisms which might incorporate free radioactivity.

The following screens were employed to remove debris from the preparations: no. 200 stainless steel screen with 80-μm pores, from C. E. Tyler, Inc., Houston, Tex., and HC10 Nitex nylon screen with a 10-μm mesh opening from TET/Kressilk Products, Inc., Houston, Tex.

Preparation of Sperm Heads

The preparation of sperm heads is based on the resistance of the sperm nucleus to digestion by a variety of agents. In the present experiments, there was no loss or damage of sperm nuclei upon sonication or chemical treatment. The nuclei were observed on a hemacytometer using phase-contrast optics.

Two to eight caudae epididymides were homogenized in 5 ml of distilled water with a motor-driven glass-Teflon homogenizer. (Samples were kept on ice for these initial steps of the preparation.) Samples were sonicated for 3 min with a Branson 20 kHz sonifier (W185, Branson Instruments Co., Stamford, Conn.) using the microprobe at a setting of 6 (25 W output). The sperm tails were broken into small pieces; nuclei and cells other than sperm heads were disintegrated. The suspensions were then filtered through an 80-μm screen, and 0.55 ml of 10% BSA in 10 mM $MgCl_2$ (pH 7.4) was added. After removal of 0.1-ml aliquots for scintillation counting, each suspension was layered over 2 ml of 3% BSA in 1 mM $MgCl_2$ (pH 7.4) in a polystyrene test tube (Falcon Plastics, Div. of B.-D. Laboratories, Inc., Los Angeles, Calif., no. 2057). The overlayering was performed to minimize contamination of the pellet by the supernate. (This step and all subsequent steps were performed at room temperature, 23° ± 1°C.) After centrifugation at 450 *g* for 20 min, the sperm heads were resuspended in 6.5 ml of a solution containing 0.9 mM $MgCl_2$, 1.7% Triton X-100, 0.19% wt/vol trypsin, and 15 μg/ml of crude DNase, and incubated for 25 min at room temperature.

The suspension was then layered over 2 ml of 5% Triton X-100 and centrifuged at 450 *g* for 20 min. The pellet was resuspended in 5 ml of distilled water, passed up and down through a 26-gauge needle three times, and filtered through a 10-μm screen. This suspension was layered over 3 ml of 5% Triton X-100 and spun at 225 *g* for 20 min.

In the initial experiments, the epididymal sperm heads were resuspended in 5 ml of 0.8% SDS (method A). In subsequent experiments, an additional step of purification was added. The sperm heads were first resuspended in 5 ml of 2 M NaCl, centrifuged at 450 *g* for 20 min, and then resuspended in SDS for counting and filtering (method B).

Electron Microscopy

The sperm heads were fixed overnight in 3% glutaraldehyde in a pH 7.4 phosphate buffer (Millonig, 1961). Samples were washed with phosphate buffer and post-fixed in buffered 1% OsO_4 for 1 h. After rapid dehydra-

tion in a graded series of ethanol, samples were embedded in Epon 812. Blocks were sectioned with a diamond knife on a Porter-Blum ultramicrotome (Ivan Sorvall Inc., Newtown, Conn.). Thin sections were taken up on 200-mesh copper grids and stained with alcoholic uranyl acetate followed by lead citrate (Reynolds, 1963). Grids were observed in a Philips 300 electron microscope operating at 40 or 60 kV.

Purification of DNA from Sperm Nuclei

Sperm heads prepared from 12 mice (by method B, omitting the SDS step) were suspended in 2.25 ml of buffer (0.1 M glycine, 0.01 M NaCl, 0.001 M EDTA, pH 9.0). One-third of the sample was removed and filtered for measurement of total radioactivity. To the remaining 1.5 ml of sample, 0.15 ml of 1 M dithiothreitol was added and the mixture incubated at 37°C for 30 min. This treatment reduces disulfide bonds and renders the sperm heads susceptible to subsequent lytic treatments (Borenfreund et al., 1961; Kistler et al., 1973). The heads were digested by adding 0.4 ml of 0.5% pronase and incubated at 37°C for 15 min. The DNA was further separated by extraction with 2 ml of phenol, followed by re-extraction of the phenol with 2 ml of glycine buffer. The aqueous phases were pooled and then split into two equal samples. The DNA was precipitated by the addition of 2 vol of cold 95% ethanol and centrifugation. The precipitates were washed with ethanol, vacuum dried, and resuspended in 1.0 ml of 0.01 M Tris-acetate buffer (pH 7.0, containing 0.01 M NaCl and 10^{-4} M EDTA). To one of the two samples, 50 μl of 60 mM $MgCl_2$ and 10 μl of pure DNase (5 mg/ml in water) were added. Both samples were then incubated at 37°C for 30 min, after which 50 μl of 10 mg/ml BSA were added as carrier and the samples were precipitated by the addition of 1.1 ml of 10% trichloroacetic acid (TCA). The precipitates were either filtered for determination of radioactivity, or centrifuged for determination of DNA by the diphenylamine reaction (Burton, 1956). The recovery of sperm DNA was about 80% (see Table IV). On the other hand, the yield of protein was less than 0.1% as indicated by [^{3}H]arginine-labeled sperm proteins, and less than 1% as determined by the method of Lowry (Lowry et al., 1951).

Radioactive Labeling and Counting

Mice were injected intraperitoneally with a total of 3 μCi/g body weight of [^{3}H]TdR or THO given in two injections. The injections were spaced 15 h apart, equal to half of the spermatogonial cell cycle (Monesi, 1962), in order more uniformly to distribute the radioactivity to avoid radiation suicide of spermatogonia (Johnson and Cronkite, 1959). Nuclear proteins of epididymal sperm were labeled by injection of 20 μCi of [^{3}H]arginine into each testis at 12–15 days before sacrifice (Lam and Bruce, 1971).

The 0.1-ml aliquots of epididymal homogenates, taken after sonication, were digested in 0.9 ml of NCS solubilizer overnight at room temperature. The sperm head suspensions, after purification, were filtered on to Whatman GF/C filters and washed with 20 ml cold distilled water, 20 ml cold 5% TCA, and 10 ml cold ethanol. The samples of purified DNA, precipitated with TCA, were filtered and washed with 20 ml cold 5% TCA and 10 ml cold ethanol. All filters were dried and samples digested in scintillation vials overnight at 37°C with 1.0 ml of NCS containing 9% water. 9 ml of scintillation fluid (4 g 2,5-diphenyloxazole, 0.05 g 1,4-bis[2-(5-phenyloxazolyl)]benzene [PPO/POPOP] per liter of toluene) were added and the samples were counted with an efficiency of 35% which showed negligible variation between samples. The background was approximately 20 cpm. The counts were proportional to the number of radioactive sperm heads on the filter at least up to 8×10^7 heads.

Because of the low levels of radioactivity in the samples, accurate determinations of background levels were necessary. Each day, three new blanks were prepared containing washed GF/C filters and scintillation fluid in vials from the same batch as used for the samples. Samples and blanks were counted for 20 min at a time and the results of several cycles of counts were averaged. This procedure minimizes variations caused by drift in the electronics of the scintillation counter. Unless otherwise noted, samples were counted for a total of 200 min to obtain statistically significant data. The 95% confidence interval for such a sample containing 5 cpm above background, determined from three background samples, is ± 0.78 cpm.

Measurement of Contaminating Radioactivity

Because of the low levels of radioactivity in the sperm head preparations, the possibility of radioactive contamination of the samples from handling must be considered. This possibility was evaluated by also preparing samples from unlabeled mice in most experiments.

Contamination by radioactivity in DNA and thymidine metabolites from other cells was also examined in these preparations. Two methods were used. In one method, nonradioactive sperm heads were purified in the presence of labeled CHO cells. These cells, obtained from Dr. R. Humphrey, were grown in monolayer cultures in modified McCoy's 5A medium supplemented with 20% fetal calf serum. CHO cells were labeled for 16 h in medium containing about 1 μCi/ml [^{3}H]TdR and then grown in nonradioactive medium for 2 h to minimize the acid-soluble pools of radioactivity. The cells were suspended by trypsinization and washed twice by centrifugation. Approximately 10^6 cells were added to 5 ml of distilled water and homogenized along with nonradioactive epididymal tissue. The sperm heads were prepared and counted as described above.

Another method for evaluating radioactive contami-

nation of sperm heads was to prepare the epididymal sperm heads from mice sacrificed at short times after injection of [^{3}H]TdR. Since the synthesis of DNA and other nuclear macromolecules is negligible in spermatozoa (Kofman-Alfaro and Chandley, 1971; Monesi, 1965), the sperm nuclei are essentially unlabeled and the radioactivity in the preparations is a measure of the upper limit of contamination.

Both methods yield a parameter, referred to as the "contamination factor." This factor is the ratio of the radioactivity in the filtered preparations of purified sperm heads to that present in the total homogenate at the start of the purification procedure. The contribution of contamination to the counts detected in any sperm head preparation may be calculated by taking the product of the counts in the total homogenate and the appropriate contamination factor.

Autoradiography

Samples of epididymal spermatozoa were prepared (from two mice each) by method B. After resuspension in SDS, half of the sample was filtered for scintillation counting and the other half was centrifuged, resuspended in 0.15 ml of 0.5% BSA, and smeared on subbed slides. The slides were dipped in Ilford K-5 emulsion, exposed for 18 days at 4°C, developed with Kodak D-19, and then stained with hematoxylin.

RESULTS

Microscope Observations of Sperm Head Preparations

Sperm nuclei were prepared from the caudae epididymides of a mouse by the methods described. An average of 2.5×10^7 and 2.0×10^7 sperm heads were present in the initial homogenate and final sample, respectively. The sperm nuclei appeared undamaged by the treatment and were free of any contaminating nuclei or cells. Only very few axial filaments of tail fragments were seen by phase-contrast microscopy in the preparations.

A typical field from a sperm head sample, prepared by method B, is shown in the electron micrograph (Fig. 1). The isolation procedure caused removal of the acrosome, plasma membrane, and nuclear envelope from sperm heads. No contamination by sperm midpieces, sperm tail fragments, or other cellular debris was observed in electron micrographs. Before treatment with SDS, small fragments of sperm tails or portions of midpieces could be seen very rarely in the preparation. However, the mitochondria of these midpieces were devoid of internal contents and only

FIGURE 1 Isolated epididymal sperm heads prepared by method B, after SDS treatment. × 15,500.

the membranes were present. Small residual membrane fragments, associated with sperm nuclei before incubation in SDS, were also eliminated by SDS treatment. The ultrastructural results indicate that the purity of the SDS-treated preparations was very high and that sperm nuclei were free of extraneous material.

Measurement of Contaminating Radioactivity

The most important criterion of purity of these preparations for the present experiments is the extent of contamination by radioactivity from other cells. To assess the magnitude of this contamination, the fractions of the radioactivity in the initial homogenates that remained on the filter with the sperm heads were measured (Table I). Contamination factors were of the order of 10^{-3} with method A. When an additional washing step was added to the procedure (method B), threefold decreases in contamination were observed. Contamination factors were not altered when the amount of tissue used was increased fourfold.

Sperm heads could be more completely separated from contaminating radioactivity from epididymal tissue than from that of labeled CHO cells. The cause of this difference may be that most of the radioactivity in the epididymis is acid soluble, but the radioactivity in CHO cells is in the form of DNA. In any case, the contamination factors obtained using CHO cells represent an upper limit of the contamination from labeled tissue at any time after injection.

Radioactivity in Sperm DNA at Various Times After [^{3}H]TdR Injection

In the first experiment, mice were injected with [^{3}H]TdR and sacrificed at 1 h and subsequent daily intervals after injection. Epididymal sperm heads were prepared by method A from individual mice. The counts of radioactivity in the homogenates and in the purified sperm head preparations are presented in Fig. 2.

The total radioactivity in the epididymal homogenate decreased steadily for 27 days. On day 28, a sharp rise in radioactivity began as radioactive sperm, labeled during S-phase, entered the cauda epididymidis. The counts reached a maximum on day 34 and then declined exponentially. The ratio of the counts in the sperm to those in the homogenate, between days 29 and 41, was 75%. This value is similar to the recovery of sperm nuclei, indicating that almost all of the radioactivity was in sperm heads.

Quantitative analysis of these kinetic data indicates that spermatozoa enter the cauda epididymidis 28.7 days after the termination of the preleptotene S-phase and remain there for an average of 6.7 days (M. L. Meistrich, manuscript in preparation). The stages of spermatogenesis at which the cells were located at the times of injection, based on this analysis and Oakberg's kinetic data (Oakberg, 1956 *b*, 1957), are presented in Table II.

The radioactivity in the sperm head preparations remained at about 5 cpm from 0 to 27 days, despite a decrease in total radioactivity in the homogenate. Between days 21 and 26, the radioactivity in sperm heads was routinely above the maximal contamination level. Thus, it appeared that radioactivity was present within the sperm nuclei at this time, but further experiments were necessary.

A second experiment was performed utilizing the improved preparation method (method B). Unlabeled mice were used, as well as mice injected at 1, 6, 11, 16, 21, 26, and 31 days before sacrifice. The levels of radioactivity in epididymal sperm head preparations were measured at various times after injection of [^{3}H]TdR (Table III). These data show that there was negligible contamination caused by handling the sample and negligible incorporation of radioactivity into spermatozoa. There was, however, significant radioactivity incorporated into cells which became spermatozoa 11–26 days after injection of [^{3}H]TdR. These cells were in the meiotic prophase or in spermiogenesis at the time of injection (Table II). Much higher levels of radioactivity were, however, found in sperm heads from mice sacrificed 31 days after injection, indicating S-phase synthesis to be much greater than meiotic DNA synthesis.

In order to determine whether the radioactivity in the sperm nucleus was in DNA, the following experiment was performed. Mice were sacrificed at 1, 6, 11, 16, 21, and 26 days after injection with [^{3}H]TdR, and three epididymal sperm head suspensions were prepared from four mice each, and pooled. One-third of the pooled sample was filtered to determine the total radioactivity in the sperm heads, one-third was used for DNA extraction, and one-third for DNA extraction followed by DNase treatment.

The radioactivity in the sperm head prepara-

TABLE I

Contamination of Epididymal Sperm Nuclei by Radioactivity in Other Cells

Source of contaminating radioactivity	Mice per preparation	Preparation method	Number of experiments	Contamination factor ± SE*
CHO Cells	1	A	5	$3.1\ (\pm 1.0) \times 10^{-3}$
CHO Cells	1	B	7	$8.7\ (\pm 2.9) \times 10^{-4}$
Epididymis‡	1	A	5	$2.8\ (\pm 0.4) \times 10^{-4}$
Epididymis‡	1	B	12	$9.1\ (\pm 1.9) \times 10^{-5}$
Epididymis‡	4	B	7	$1.2\ (\pm 0.3) \times 10^{-4}$
Epididymis§	4	B	6	$7.5\ (\pm 1.0) \times 10^{-5}$

* Radioactivity in filtered sperm head preparation ÷ radioactivity in homogenate.

‡ The epididymides of mice injected with [^{3}H]TdR 1 day before sacrifice were used.

§ Epididymides were obtained 6 days after [^{3}H]TdR injection.

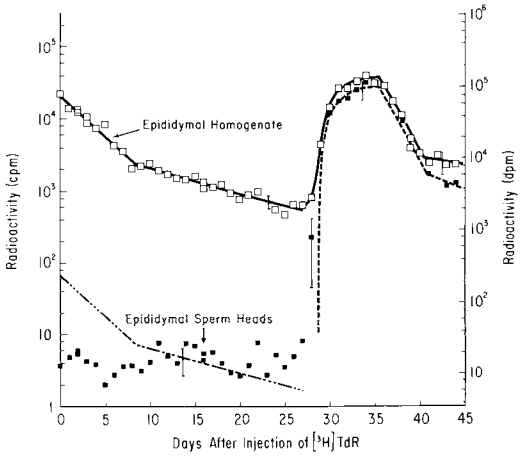

FIGURE 2 Radioactivity in epididymal homogenates (open symbols, solid lines) and sperm head preparations (filled symbols, dashed lines) from mice sacrificed at various times after injection of [^{3}H]TdR. The time interval was measured from the second of two injections given 15 h apart. Each point represents the average of three samples, each prepared from an individual mouse and scintillation counted for 40 min. Error bars represent the average experimentally determined 95% confidence intervals for each point. (These intervals are proportional to the mean and, hence, the bars would have the same length anywhere along the logarithmically plotted curves.) Where the confidence intervals differed between the regions of the curves, additional error bars are given. Exponential curves were calculated, when appropriate, using a least squares exponential fit. The alternately dotted and dashed line represents the maximum contribution of contamination to the radioactivity in the sperm head preparations (i.e., contamination factor times radioactivity in homogenate).

tions (first column of Table IV) generally confirmed the previous experiment (Table III). The radioactivity in the 1-day sample was higher than expected from extrapolation of the data of Table III. This is partly explained by the observation that when epididymides of four mice rather than one mouse were used in each preparation, the radioactivity in the homogenates was increased eightfold; this might be caused by the more vigorous homogenization used with the larger amount of tissue. Nevertheless, the low levels of radioactivity in the samples prepared on day 6 demonstrate that contamination of the sperm heads by radioactivity in the homogenates is minimal at postinjection times longer than 6 days.

Significant amounts of DNase-sensitive radioactivity were observed in all preparations from labeled mice. The radioactive DNA present on days 1 and 6 may represent contamination from epididymal cells. At 11 and 16 days after injection, radioactivity in DNA represented less than 0.5 of the radioactivity in the sperm heads. On day 21 and especially on day 26, the fractions of radioactivity in DNA were higher. When corrected for the 79% average recovery of DNA in this procedure, these values demonstrate that more than 0.5 of the total radioactivity in the sperm head was in DNA.

To determine whether the radioactivity observed was incorporated directly from [^{3}H]TdR or involved THO as an intermediate, we injected different groups of mice with equal amounts of radioactivity in the form of [^{3}H]TdR or THO. Mice were sacrificed at 1, 6, 11, 16, 21, 26, and 34 days after injection and samples of sperm heads (four mice per sample) were prepared by method B (Table V).

The radioactivity in sperm heads from THO-injected mice represents an upper limit on the amount of incorporation in the TdR-injected mice which could involve THO as an intermediate.

TABLE II

Stages of Differentiation of Sperm Cells in Cauda Epididymidis at Time of [^{3}H]TdR Labeling

Days between second injection of [^{3}H]TdR and sacrifice	Stage of cells at time of injection*
1	Epididymal spermatozoa
6	Epididymal spermatozoa
11	Spermatids 8–16‡
16	Spermatids 1–11
21	Pachytene (VII)-spermatid 4§
26	Zygotene-pachytene (X)§
28	Leptotene-pachytene (VII)§
31	Spermatogonia B-pachytene (II)§

* Two injections of [^{3}H]TdR were given 15 h apart. The ranges of cell stages presented in the table include those stages which the sperm heads were in for at least one of the two injections.

‡ Arabic numerals indicate the steps of spermatid development.

§ Roman numerals indicate the stages of the cycle of the seminiferous epithelium.

Levels of radioactivity present in sperm heads prepared 11–26 days after injection of either [^{3}H]TdR or THO were significantly higher than those in sperm heads prepared on days 1 and 6. The incorporation of tritium from THO was highest on days 11 and 16, and could account for most but significantly not all of the incorporation from [^{3}H]TdR in these cell stages. At 21 and 26 days after injection , only a small proportion of the incorporation from [^{3}H]TdR could involve a pathway which included THO. The minimal amount of incorporation of THO into DNA is emphasized by the measurement taken at 34 days representing cells which were in S-phase when the isotope was injected.

Autoradiographic Analysis

It is not possible to determine by liquid scintillation counting whether the low levels of radioactivity measured in spermatozoan nuclei represent uniform low-level labeling of all cells or the presence of a small number of S-phase cells. The possibility of finding cells labeled as preleptotene spermatocytes in the cauda epididymidis on days 21 and 26 after injection was rendered unlikely by quantitative analysis of the kinetic data and was experimentally tested by autoradiography. In au-

TABLE III

Radioactivity Present in Mouse Epididymal Sperm Heads Prepared by Method B at Various Times After Injection of [^{3}H]TdR (3 μCi/g Body Weight)

Days between second injection of [^{3}H]TdR and sacrifice	Number of samples	Average radioactivity in sperm heads from one mouse ± SE	Maximum contribution of contaminating radioactivity from epididymal homogenate*
		cpm	*cpm*
Unlabeled	17	0.2 ± 0.3	—
1	13	1.4 ± 0.3	13.9
6	9	1.8 ± 0.4	3.7
11	12	3.4 ± 0.7‡	1.6
16	12	2.4 ± 0.4§	1.1
21	14	3.2 ± 0.5‡	0.8
26	11	5.5 ± 0.9‡	0.5
31	3	$1.33 \times 10^4 \pm 0.11 \times 10^4$	—

In this experiment only, occasional samples showed higher levels of contamination. Therefore, the mean and standard error were computed after excluding outlying points which deviate from a normal distribution at the 1% level of significance (Swaroop, West and Lewis, 1969).

* Calculated by multiplying the maximum contamination factor obtained using CHO cells (8.7×10^{-4}) by the radioactivity in the epididymal homogenate.

‡ Radioactivity levels significantly different from 1- and 6-day values at $p < 0.01$.

§ Radioactivity levels significantly different from 1- and 6-day values at $p < 0.05$.

TABLE IV

Radioactivity in Mouse Epididymal Sperm Nuclei and in DNA Extracted from These Nuclei at Various Times After Injection of [³H]TdR (3 μCi/g Body Weight) and Relative DNA and Protein Contents of the Preparations

Days between second injection of [³H]TdR and sacrifice	Average radioactivity in sperm heads from four mice (cpm)*			Fraction of radioactivity in sperm heads recovered in DNA preparation
	Intact sperm heads	Purified DNA	DNase-treated DNA preparation	
Unlabeled	0.3	0.4	0.3	—
1	14.5	5.9	0.1	0.40
6	2.3	1.6	−0.1	~0.7
11	14.2	3.5	0.0	0.25
16	13.4	3.9	0.0	0.29
21	9.5	3.9	0.4	0.41
26	16.6	9.5	0.1	0.57
Radioactivity 35–41 days after [³H]TdR injection‡	100%	69%	0.22%	0.69
DNA recovery using diphenylamine reaction§	100%	89%	1.3%	(0.89)
Radioactivity 12–15 days after [³H]arginine injection	100%	0(−0.1%)	0(−0.06%)	0

* Each value represents the average of two experiments.

‡ Values are given as percentage of total radioactivity in aliquot of intact sperm heads.

§ Values given as percentage of DNA recovered.

TABLE V

Radioactivity Present in Mouse Epididymal Sperm Heads at Various Times After Injection of [³H]TdR or THO (3 μCi/g Body Weight)

Days between second injection of isotope and sacrifice	Average radioactivity in sperm heads from four mice ± SE*		Radioactivity incorporated from THO
	[³H]TdR injection	THO injection	Radioactivity incorporated from TdR
	cpm	*cpm*	
Unlabeled		(0.3 ± 0.3)	
1	11.1 ± 1.2	0.4 ± 0.8	0.04
6	5.0 ± 0.4	1.7 ± 0.4	0.35
11	17.2 ± 1.8	13.6 ± 0.5	0.79
16	20.7 ± 1.3	14.5 ± 2.2	0.70
21	14.0 ± 1.2	5.5 ± 0.9	0.39
26	22.0 ± 0.2	5.4 ± 0.5	0.25
34	1.1×10^5	9.9	10^{-4}

* Each value represents four samples of four mice each; except for the unlabeled and 34-day values which represent 12 and 1 sample, respectively.

toradiographs, uniform labeling of the sperm nuclei should be undetectable, but a small number of S-phase nuclei should be observable.

Samples of epididymal sperm heads were prepared and each sample was processed both for autoradiography and for scintillation counting (Table VI). Among 75,000 sperm heads scored on smears from mice sacrificed on days 21 and 26, only a few lightly labeled nuclei were observed, consistent with background levels determined on smears of sperm from unlabeled mice. In the 21- and 26-day samples no nuclei were found which

TABLE VI

Autoradiographic Analysis of Radioactivity in Mouse Epididymal Sperm Head Preparations After Injection of [^{3}H]TdR (3 μCi/g Body Weight)

Days between (second) injection of [^{3}H]TdR and sacrifice	Radioactivity in sperm heads of one mouse (scintillation counting)	Numbers of nuclei counted on autoradiographs				Statistical analysis	
		Total counted	2–4 grains	5–7 grains	≥8 grains (S-phase)	Expected number of S-phase nuclei*	*P* (obs = exp)‡
	cpm						
Unlabeled	1.0	75,000	94	2	0	—	—
Unlabeled	0.4	75,000	75	2	0	—	—
21 days	3.8	75,000	67	0	0	7.9	<0.001
26 days	2.1	75,000	49	2	0	4.3	0.01
Unlabeled + 33 days§	5.4	75,000	71	1	10	11.3	0.42
33 days	2.2×10^4	750	11	13	460	—	—

* Calculated from the ratio of the radioactivity in the sample to that in the 33-day sample by the formula: Expected number of S-phase nuclei = $[cpm/(2.2 \times 10^4)] \times (460/750) \times 75{,}000$; which is based on the assumption that the radioactivity is all concentrated in S-phase cells.

‡ Probability, based on Poisson statistics, that a deviation from the expected value equal to or greater than that observed could occur by chance.

§ A small sample of sperm heads from the 33-day sample was added to the second unlabeled sample in the ratio of 1:5,000.

could have been in S-phase at the time of injection, as were routinely observed on smears of sperm from mice sacrificed at 33 days postinjection. To ascertain that few S-phase nuclei could indeed be identified in the presence of a vast excess of unlabeled nuclei, smears of a sample of unlabeled sperm heads, containing one part in 5,000 of the 33-day sample, were examined. The expected number of labeled sperm nuclei was indeed observed. Thus, we must conclude that the radioactivity in sperm nuclei of mice sacrificed 21 and 26 days after injection of [^{3}H]TdR cannot be in a small number of cells which were highly active in DNA synthesis, but probably represents uniform labeling of all cells at a very low level.

DISCUSSION

Meiotic DNA synthesis has been examined in a variety of plants and animals, but there is no general agreement on its ubiquitous existence. The most convincing demonstration of meiotic DNA synthesis is based on biochemical analysis of unique density DNA synthesized during zygotene and pachytene in the lily (Hotta, Ito and Stern, 1966; Hotta and Stern, 1971). It has, however, been difficult to demonstrate this DNA synthesis autoradiographically (Taylor and McMaster, 1954; Taylor, 1959; Ito and Hotta, 1973). Meiotic DNA synthesis has been reported in other plants as well (Riley and Bennett, 1971; Flavell and Walker, 1973).

In animals, meiotic DNA synthesis in vivo has been reported in a newt (Wimber and Prensky, 1963), but with the same methods no synthesis could be detected in a different species of newt (Callan and Taylor, 1968). Numerous studies employing in vivo injection of [^{3}H]TdR into mice, but with one exception (Mukherjee and Cohen, 1968), have failed to detect any significant labeling of meiotic cells by autoradiographic methods (for example, Monesi, 1962; Clermont and Trott, 1969; Odartchenko and Pavillard, 1970; Kofman-Alfaro and Chandley, 1970).

Several workers (Kofman-Alfaro and Chandley, 1971; Chandley and Kofman-Alfaro, 1971; Lima de Faria et al., 1968) have shown incorporation of [^{3}H]TdR into DNA of untreated male meiotic cells during in vitro incubation. Since mammalian germinal cells fail to differentiate through the meiotic prophase in cell culture, and even in organ culture fail to undergo the meiotic divisions (Steinberger et al., 1970), the possibility exists that the in vitro incorporation of [^{3}H]TdR is a response to cell damage occurring during preparation of suspensions or in vitro incubation and not a measure of a normal in vivo process.

In this study, we have employed in vivo labeling and purification of sperm nuclei to demonstrate meiotic DNA synthesis. The synchrony of spermatogenesis has permitted the collection of epididymal sperm nuclei which were in precise stages of spermatogenesis at the time of labeling. Very low, but nevertheless significant levels of radioactivity were present in the sperm nuclei prepared from the epididymis at several intervals after injection so that the cells were either in the meiotic prophase or spermiogenesis at the time of injection. Four separate experiments (Tables III, IV, V, and VI) confirmed this observation. The increased radioactivity in sperm nuclei 11–26 days after injection over the level seen at 6 days, despite a decline in total epididymal radioactivity, rules out the possibility that contamination by epididymal cells is responsible for the counts.

Liquid scintillation counting was employed for measurement of this radioactivity since it is much more sensitive than autoradiography. We have measured radioactivity levels of 3×10^{-7} dpm per sperm nucleus. The sensitivity of autoradiography is estimated to be only 3×10^{-4} dpm/cell by the following parameters: efficiency of 0.05 grain per disintegration (Rogers, 1967), two grains per cell to indicate labeling above background, exposure time limit of 3 mo to minimize fading of latent images and increases in background.

It was necessary to determine that the incorporation of radioactivity into the sperm heads proceeded directly from thymidine into DNA. Tritium from thymidine can be incorporated at low levels into other macromolecules such as proteins (Bryant, 1966), presumably by a transmethylation reaction or catabolism to THO (Rubini et al., 1960) and reutilization in biosynthetic reactions. As DNA comprises nearly half of the sperm nucleus, this problem is minimized. The data of Table IV show that most of the radioactivity incorporated into meiotic cells was in DNA. On the other hand, when cells were in spermiogenesis at the time of [^{3}H]TdR injection, most of the incorporation was into other components, presumably the major sperm nucleoproteins which are synthesized in the elongating spermatids (Monesi, 1965; Lam and Bruce, 1971).

Incorporation of tritium into macromolecules does not necessarily represent de novo synthesis from labeled precursors. For example, tritium can be incorporated by "hydrogen" exchange from THO (Printz and von Hippel, 1968). Table V shows that water is not an intermediate in the incorporation of tritium into meiotic cells, but may be an intermediate in the spermatids, in which tritium from thymidine may be incorporated into protein. The possibility that [^{3}H]TdR is acting as a methyl group donor and labeling DNA by transmethylation has not been tested and remains an alternative explanation of the results.

It is unlikely that the incorporation of [^{3}H]TdR into DNA of meiotic cells is a result of repair of damage caused by the administered radioactivity (Painter and Young, 1974). After injection, the radioactivity levels were 4×10^5 dpm per testis resulting in only 0.03 rads of internal radiation during the 1-h in vivo lifetime of the [^{3}H]TdR (Cleaver, 1967). We have demonstrated in other experiments that no measurable increase in [^{3}H]TdR incorporation into meiotic cells occurs after 100 rads of X irradiation.

We wish to estimate the relative levels of DNA synthesis in the meiotic and the S-phase cells from the present data. We are aware that the incorporation of labeled precursors into various cells can be affected by differences between the cells in accessibility to the label, in transport through the cell membrane, in kinase levels, and in intracellular pool sizes, and therefore, may not quantitatively represent the levels of DNA synthesis. Hence, all quantitative comparisons will be made with the assumption that these parameters do not differ widely between cell types.

The incorporation of [^{3}H]TdR into spermatogenic cells in the later stages could conceivably be limited by the penetration of the isotope, perhaps by the blood-testis barrier. The barrier at the peritubular myoid cells would not alter the ratio of label reaching the spermatogonia to that reaching the spermatocytes. Within the seminiferous tubules, the Sertoli cell processes separate the germinal epithelium into a basal compartment containing spermatogonia and preleptotene spermatocytes, and an adluminal compartment containing meiotic spermatocytes and spermatids (Dym and Fawcett, 1970). This separation constitutes a barrier to the penetration of proteins and small particulates with dimensions in the 20–60 Å range (Dym and Fawcett, 1970; R. Vitale, personal communication). Smaller molecules, however, do penetrate quite freely into cells in the adluminal compartment, as demonstrated by labeling with radioactive amino acids and uridine (Monesi, 1965; Lam and Bruce, 1971; Meistrich, 1972), binding of actinomycin D (Barcellona et al., 1974), and the greater effect of certain chemical mutagens

on cells in later stages of spermatogenesis (Ehling, 1971). Thus, although we have no direct evidence that [^{3}H]TdR is able freely to penetrate the blood-testis barrier, previous studies indicate that such small molecules do reach all of the spermatogenic cells.

The percentage of the genome synthesized during meiosis in *Lilium* was computed by Hotta et al., (1966, 1971) from specific activities of the meiotic DNA and from DNA hybridization studies to be about 0.4%. In our experiments, the radioactivity in epididymal sperm heads from one mouse, resulting from the primary spermatocytes in S-phase at the time of labeling, is calculated to be 1.5×10^4 cpm and corresponds to replication of one diploid genome per S-phase cell. The heads prepared 26 days after injection represent cells labeled during a 6.7-day interval of the meiotic prophase (Table II) and contain 3 cpm in DNA. Thus, the total incorporation during the 10-day meiotic prophase would be about 5 cpm, corresponding to the synthesis of about 0.03% of the diploid genome. This value is about an order of magnitude lower than that observed in the lily.

For comparison with autoradiographic data, the appropriate parameter is the ratio of the rates of [^{3}H]TdR incorporation into meiotic and S-phase cells. The rate of incorporation of radioactivity into the preleptotene spermatocytes is 1.5×10^4 cpm per 14-h S-phase or 2.6×10^4 cpm per day. The average meiotic rate of tritium incorporation is 3 cpm per 6.7 days or 0.5 cpm per day, which is 0.002% of the rate of S-phase synthesis. This ratio is nearly three orders of magnitude below that reported for in vivo incorporation into newt testis cells (Wimber and Prensky, 1963). Quantitative comparison with the in vitro data of Kofman-Alfaro and Chandley (1971) is not possible since those authors did not report grain counts of S-phase cells.

This study has demonstrated that a small amount of DNA synthesis occurs during the meiotic prophase of mouse spermatogenesis. At present, there is no basis for speculation as to whether this synthesis is involved in repair, recombination, or some other process. The low background levels of such "unscheduled" DNA synthesis during meiosis should be quite useful for studies of repair synthesis after treatment with certain mutagenic agents in vivo as has previously been demonstrated in vitro (Chandley and Kofman-Alfaro, 1971; Gledhill and Darzynkiewicz, 1973).

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