Paper presented at the Banbury Conference on "Indicators of Genotoxic Exposure in Man and Animals," Cold Spring Harbor, N.Y., April 18-21, 1982

DNA Repair in Spermatocytes and Spermatids of the Mouse*

Gary A. Sega

Biology Division, Oak Ridge National Laboratory, Oak Ridge, TN 37830

---

*Research sponsored by the Office of Health and Environmental Research, U.S. Department of Energy, under contract W-7405-eng-26 with the Union Carbide Corporation.

Send proof to: Dr. Gary A. Sega

Biology Division

Oak Ridge National Laboratory

P. O. Box Y

Oak Ridge, TN 37830

phone (615) 574-0848

Running head: DNA repair in the mouse

Introduction

The first evidence for unscheduled DNA synthesis (UDS) in mammalian somatic cells was provided by Rasmussen and Painter, 1964, when they demonstrated that U.V. radiation induced the uptake of labeled thymidine into the DNA of cultured HeLa and Chinese hamster cells grown in culture. Later, Kofman-Alfaro and Chandley, 1971, also using in vitro procedures, were able to demonstrate UDS in spermatogenic cells of the mouse after exposure to X rays or U.V. Similar findings were observed in rat germ cells (Gledhill and Darzynkiewicz, 1973) and in human germ cells (Chandley and Kofman-Alfaro, 1971) after in vitro U.V. exposure.

The induction of UDS in mammalian germ cells can also be studied by in vivo procedures. In the male mouse it is possible to study UDS in vivo in meiotic and post-meiotic germ-cell stages (primary spermatocytes through mature spermatozoa) by making use of the sequence of events that occurs during spermatogenesis and spermiogenesis. In the developing germ cells of the mouse the last scheduled DNA synthesis takes place during a 14-h period in pre-leptotene spermatocytes (Monesi, 1962). After DNA synthesis these spermatocytes continue to develop through a series of germ cell stages for about a 28- to 30-day time period before leaving the testis as late spermatids (Oakberg, 1956a, b). After leaving the testis, the late spermatids (now immature spermatozoa) enter the caput epididymis. Two to three days later they reach the caudal epididymis and in two to three days more they reach the vas as functional spermatozoa (See Fig. 1). (F-1)

If the DNA from any meiotic or post-meiotic germ-cell stage is damaged by a physical or chemical agent and UDS is induced, it can be detected by an unscheduled incorporation of [^{3}H]dThd into the affected germ cells. The

unscheduled uptake of [^{3}H]dThd can be measured either directly, by examining the affected germ-cell stages through autoradiography (Sotomayor et al., 1979), or indirectly, by waiting until the germ cells have matured into sperm in the caudal epididymis and vas, and then assaying the [^{3}H]dThd activity contained in several million of these sperm by using liquid scintillation counting (Sega, 1974; Sega et al., 1976, 1978).

Methods

Male mice are exposed to a chemical or physical agent in exactly the same way they would be in a genetic experiment. The general method of administration of chemicals is by intraperitoneal (i.p.) injection, but other routes such as inhalation, gavage and direct testicular injections have also been used (Sega and Sotomayor, 1982; G. A. Sega, unpublished data). [^{3}H]dThd is injected directly into the testes either at the same time or at different times after mutagen treatment. For the testicular injections the mice are anesthetized with Metofane, a small incision is made in the scrotum to visualize the testes, and 36 µl of water containing ~18 to 36 µCi of [^{3}H]dThd is injected into each testis. The incision heals in a few days without any special treatment.

To make direct observations of the germ-cell stages undergoing UDS, autoradiographic procedures are used (Sotomayor et al., 1979; Sega and Sotomayor, 1982). These techniques also provide information on the uniformity of the UDS response within a particular germ-cell stage and can be used to study the distribution of [^{3}H]dThd labeling from UDS throughout the chromosomes in diakinesis. However, autoradiographs are hard to

quantitate and are not as sensitive as LSC for detecting low levels of induced UDS.

UDS occurring in any germ-cell stage can be studied indirectly by making use of the timing of spermatogenesis and spermiogenesis in the mouse and recovering sperm from the caudal epididymis or vas at the appropriate time after treatment. For example, sperm recovered from the caudal epididymis 16 days after treatment represent germ-cells that were mostly in early spermatid stages at the time of treatment. If a "mutagen exposure - UDS response" curve is desired for a particular germ-cell stage, then at the appropriate time after each level of exposure the mice are killed and sperm are recovered from the caudal epididymis or vas. If it is desired to study all of the meiotic and post-meiotic germ-cell stages undergoing a UDS response to a particular agent, sperm samples are recovered from treated animals every few days for 4-5 weeks after treatment.

For sperm recovery, the caudal epididymides or vasa are dissected from the animals, diced, and sonicated to destroy all cell types except for the sperm heads. The tails and midpieces are sheared off in this process. After several washes in a sucrose-SDS solution, a pure population of sperm heads is obtained free of sperm tails, midpieces, somatic cells, or cellular debris (Sega, 1974; Sega et al., 1976; Sega and Sotomayor, 1982). Typically, 7×10^6 sperm heads are recovered from the vas of one male and about twice that number are recovered from the caudal epididymis. Several million of these sperm are assayed by LSC to determine [^{3}H]dThd activity, and the number of sperm present in each scintillation vial is determined by hemacytometer counts of a diluted sample of the same sperm stock. Finally, the [^{3}H]dThd dpm/10^6 sperm heads is calculated. Although LSC is an

indirect means of measuring the UDS that had occurred in various meiotic and post-meiotic germ cell stages, it has the advantage of sensitivity to low levels of induced UDS since millions of sperm heads are sampled.

Results and Discussion

Germ-Cell Stages that Undergo a UDS Response to Mutagenic Agents

In our studies that looked at all of the meiotic and post-meiotic germ-cell stages undergoing a UDS response to mutagen treatment we have used methyl methanesulfonate (MMS), ethyl methanesulfonate (EMS), cyclophosphamide, mitomen and X rays (Sega, 1974; Sega et al., 1976; Sotomayor et al., 1978; 1979). Other workers have studied UDS induced by X rays and U.V. light in an in vitro system with mouse germ cells (Kofman-Alfaro and Chandley, 1971), U.V. in vitro with rat germ cells (Gledhill and Darzynkiewicz, 1973), and MMS and procarbazine (Schmid et al., 1978; Burgin et al., 1979) in vivo with the rabbit. In all of these studies the basic observation is that mutagens can induce UDS in meiotic stages and post-meiotic stages up to about mid-spermatids. No UDS is detected in late spermatids and sperm cells.

The absence of UDS in the late spermatid stages to mature spermatozoa treated with MMS and EMS is not due to failure of these chemicals to alkylate DNA in these stages. Our chemical dosimetry studies have shown that DNA in these germ-cell stages is being alkylated (Sega et al., 1974; Sega and Owens, 1978; G. A. Sega, unpublished data). As we have discussed previously (Sega, 1974), the germ cell stages failing to exhibit a UDS response are those in which protamine has either replaced, or is in the process of replacing the chromosomal histones. At the time of protamine

synthesis an extensive condensation of the spermatid nucleus begins. It is possible that the DNA lesions present in these cells have become inaccessible to the enzymatic system which gives rise to UDS in the earlier germ-cell stages. Also, much of the cytoplasm is lost from the spermatids as they develop from mid to late spermatid stages, and the enzymatic system which produces the UDS response may be lost at this time.

UDS Response With Different Test Agents

A number of agents which have been tested for their ability to induce a UDS response in the germ cells of male mice are shown in Table 1. (T-1) Generally, if a mutagen produces a positive UDS response in mouse germ cells, the UDS can be detected using lower exposures than those required to measure genetic endpoints. Also, when exposures to a test agent are below toxic levels, there is usually a linear relationship between the exposure level and the UDS response of the germ cells (Sega et al., 1976; 1978; 1981). Fig. 2 shows an example of the linear relationship obtained between i.p. (F-2) exposures to MNU and the UDS response observed in early spermatid stages of the mouse. Such "chemical exposure - UDS response" curves can be an aid in determining how reasonable it is to linearly extrapolate genetic results obtained at high exposure levels to what would be expected at low exposure levels.

Not all mutagens tested have given a positive UDS response in the germ cells. For example, DMN and DEN both require metabolic activation in tissues such as liver, kidney, and lung to be biologically active (Weekes and Brusick, 1975). The inability of these chemicals to induce a measurable UDS response in mouse germ cells may be explained by the low levels of mixed function oxidase activity found in the testes (Magee and

Barnes, 1967) and by the failure of active metabolites, derived from other tissues, to reach the testes.

B[a]P also requires metabolic conversion to be biologically active. We have found that after i.p. injection of ^{3}H-labeled B[a]P dissolved in corn oil, the B[a]P is present to the same extent in testis homogenates as it is in liver homogenates from the same treated animals. However, there is no clearly demonstrable binding of B[a]P (or any of its metabolites) to sperm DNA or testicular DNA, while there is extensive binding of ^{3}H-labeled metabolites of B[a]P to liver DNA (See Fig. 3). Failure to detect binding of B[a]P or its metabolites to testicular DNA is in agreement with our negative UDS results with this compound. F-3

Two other chemicals, that are currently of great environmental interest, ETO and EDB, have also been studied for their ability to induce a UDS response in mouse germ cells (See Table 1). ETO is an important industrial chemical and is widely used as a sterilant and fumigant. EDB is used as a gasoline additive and is also used extensively as a pesticidal fumigant. Recently, it has been used to help control the Mediterranean fruit fly in California (Walsh, 1982).

After inhalation exposure to ETO, UDS is induced in mouse germ cells (Cumming and Michaud, 1979). Chemical dosimetry experiments using ^{3}H-labeled ETO have also been carried out (G. A. Sega and R. B. Cumming, unpublished data). The dosimetry experiments show a gradual removal of alkylation products from testicular DNA with time after inhalation exposure to 3 ppm-h of [^{3}H]ETO (See Fig. 4). By 4 days after exposure only about 10% of the adducts remain in the testicular DNA. Thus, with ETO, there is F-4

agreement between the positive UDS response of the germ cells and removal of adducts from testicular DNA.

As Table 1 and Fig. 5 indicate, EDB does not induce a measurable UDS response in mouse germ cells. Our chemical dosimetry experiments carried out using [^{3}H]EDB have shown that after exposing mice to EDB, DNA adducts are formed in liver, testis and sperm (See Fig. 6). While adducts are gradually removed from liver DNA, no measurable removal of adducts from testicular DNA is observed over at least a 12-day period following exposure. In the case of EDB there is also agreement between the negative UDS response of the germ cells and the persistence of DNA adducts in the testis.

F-5

F-6

Relation Between UDS and Repair of Genetic Damage

Because not all germ-cell stages in the mouse undergo a UDS response after mutagen exposure, it has been possible to use this fact to study what effect UDS may have in altering the ultimate expression of genetic damage. The results to date have been ambiguous. Chemicals such as ethyl methanesulfonate (EMS) and methyl methanesulfonate (MMS) produce dominant lethals and translocations only in mid to late spermatids and spermatozoa, where no UDS occurs (Generoso and Russell, 1969; Ehling et al., 1968). In germ cell stages where UDS is induced by these chemicals no dominant lethals or translocations are induced. However, mutagens such as isopropyl methanesulfonate (iPMS) and X rays produce dominant lethals in germ cell stages where UDS has been induced (Ehling, 1971; Ehling et al., 1972; Schröder and Hug, 1971). In fact, with X rays, the dominant lethal frequency is about twice as high in postgonial germ cell stages where UDS occurs as it is in the more advanced stages where no UDS can be detected.

With X rays, there is also no reduction in specific locus mutation frequencies in postgonial germ cell stages where UDS occurs compared to those postgonial stages in which no UDS occurs (Sega et al., 1978). Clearly, much more work will be required to understand the relationship between UDS and the repair of genetic damage in mammalian germ cells.

Summary

When male mice are exposed to chemical agents that reach the germ cells several outcomes are possible in terms of the germ cell UDS response and removal of DNA adducts. It is possible that:

1. The chemical binds to the DNA and induces a UDS response with concomittant removal of DNA adducts (e.g., ETO).
2. The chemical binds to the DNA but no UDS response is induced (e.g., EDB).
3. The chemical does not bind to DNA and no UDS is induced (e.g., B[a]P).

Many mutagens have already been shown to induce a UDS response in postgonial germ cell stages of the male mouse up through about mid-spermatids, but the relationship between this UDS and the repair of genetic damage within the germ cells is still unknown. While some mutagens appear to have an effect only in germ-cell stages where no UDS occurs, others are able to induce genetic damage in stages where UDS has been induced.

References

Bürgin, H., B. Schmid, and G. Zbinden. 1979. Assessment of DNA damage in germ cells of male rabbits treated with isoniazid and procarbazine. Toxicology 12:251.

Chandley, A. C. and S. Kofman-Alfaro. 1971. "Unscheduled" DNA synthesis in human germ cells following UV irradiation. Exp. Cell Res. 69:45.

Cumming, R. B. and T. A. Michaud. 1979. Mutagenic effects of inhaled ethylene oxide in mice. Environ. Mut. 1:166.

Ehling, U. H. 1971. Comparison of radiation and chemically-induced dominant lethal mutations in male mice. Mutation Res. 11:35.

Ehling, U. H., R. B. Cumming, and H. V. Malling. 1968. Induction of dominant lethal mutations by alkylating agents in male mice. Mutation Res. 5:417.

Ehling, U. H., D. G. Doherty and H. V. Malling. 1972. Differential spermatogenic response of mice to the induction of dominant lethal mutations by n-propyl methanesulfonate and isopropyl methanesulfonate. Mutation Res. 15:175.

Generoso, W. M. and W. L. Russell. 1969. Strain and sex variations in the sensitivity of mice to dominant lethal induction with ethyl methanesulfonate. Mutation Res. 8:589.

Gledhill, B. L. and Z. Darzynkiewicz. 1973. Unscheduled synthesis of DNA during mammalian spermatogenesis in response to UV irradiation. J. Exp. Zool. 183:375.

Kofman-Alfaro, S. and A. C. Chandley. 1971. Radiation-initiated DNA synthesis in spermatogenic cells of the mouse. Exp. Cell Res. 69:33.

Magee, P. N. and J. M. Barnes. 1967. Carcinogenic nitroso compounds. Adv. Cancer Res. 10:163.

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.

Oakberg, E. F. 1956a. A description of spermiogenesis in the mouse and its use in analysis of the cycle of the seminiferous epithelium and germ cell renewal. Am. J. Anat. 99:391.

Oakberg, E. F. 1956b. Duration of spermatogenesis in the mouse and timing of stages of the cycle of the seminiferous epithelium. Am. J. Anat. 99:507.

Rasmussen, R. E. and R. B. Painter. 1964. Evidence for repair of ultraviolet damaged deoxyribonucleic acid in cultured mammalian cells. Nature 203:1360.

Schmid, B., I. P. Lee, and G. Zbinden. 1978. DNA repair processes in ejaculated sperm of rabbits treated with methyl methanesulfonate. Arch. Toxicol. 40:37.

Schröder, J. H. and O. Hug. 1971. Dominante Letalmutationen in der Nachkommenschaft bestrahlter männlicher Mäuse. I. Untersuchung der Dosiswirkungsbeziehung und des Unterschiedes zwischen Ganz- und Teilkorperbestrahlung bei meiotischen und postmeiotischen Keimzellenstadien. Mutation Res. 11:215.

Sega, G. A. 1974. Unscheduled DNA synthesis in the germ cells of male mice exposed in vivo to the chemical mutagen ethyl methanesulfonate. Proc. Natl. Acad. Sci. (USA) 71:4955.

Sega, G. A. and R. E. Sotomayor. 1980. Chemical dosimetry and unscheduled DNA synthesis studies of ethylene dibromide in the germ cells of male mice. Environ. Mut. 2:274.

Sega, G. A. and R. E. Sotomayor. 1982. Unscheduled DNA synthesis in mammalian germ cells. -- Its potential use in mutagenicity testing. In Chemical Mutagens - Principles and Methods for their Detection (ed. F. J. de Serres and A. Hollaender), 7:421, Plenum, New York.

Sega, G. A., R. B. Cumming and M. F. Walton. 1974. Dosimetry studies on the ethylation of mouse sperm DNA after in vivo exposure to [^{3}H]ethyl methanesulfonate. Mutation Res. 24:317.

Sega, G. A., J. G. Owens, and R. B. Cumming. 1976. Studies on DNA repair in early spermatid stages of male mice after in vivo treatment with methyl-, ethyl-, propyl-, and isopropyl methanesulfonate. Mutation Res. 36:193.

Sega, G. A., R. E. Sotomayor, and J. G. Owens. 1978. A study of unscheduled DNA synthesis induced by X-rays in the germ cells of male mice. Mutation Res. 49:239.

Sega, G. A., K. W. Wolfe, and J. G. Owens. 1981. A comparison of the molecular action of an S_N1-type methylating agent, methyl nitrosourea and an S_N2-type methylating agent, methyl methanesulfonate, in the germ cells of male mice. Chem.-Biol. Interactions 33:253.

Sotomayor, R. E., G. A. Sega, and R. B. Cumming. 1978. Unscheduled DNA synthesis in spermatogenic cells of mice treated in vivo with the indirect alkylating agents cyclophosphamide and mitomen. Mutation Res. 50:229.

Sotomayor, R. E., G. A. Sega, and R. B. Cumming. 1979. An autoradiographic study of unscheduled DNA synthesis in the germ cells of male mice treated with X-rays and methyl methanesulfonate. Mutation Res. 62:293.

Walsh, J. 1982. Spotlight on pest reflects on pesticide. Science 215:1592.

Weekes, U. and D. Brusick. 1975. In vitro metabolic activation of chemical mutagens. II. The relationships among mutagen formation, metabolism and carcinogenicity for dimethylnitrosamine and diethylnitrosamine in the livers, kidneys and lungs of BALB/cJ, C57BL/6J and RF/J mice. Mutation Res. 31:175.

Table 1. Agents tested for their ability to induce unscheduled DNA synthesis (UDS) in Mouse Germ Cells

AGENT	EXPOSURE	UDS RESPONSE[a]
methyl methanesulfonate (MMS)[b]	5-100 mg/kg, i.p.	+
ethyl methanesulfonate (EMS)[b]	10-300 mg/kg, i.p.	+
propyl methanesulfonate (PMS)[b]	50-700 mg/kg, i.p.	+
isopropyl methanesulfonate (iPMS)[b]	10-170 mg/kg, i.p.	+
cyclophosphamide (CPA)[c]	200 mg/kg, i.p.	+
mitomen (DMO)[c]	40 mg/kg, i.p.	+
ethylene oxide (ETO)[d]	300-500 ppm × 8 h	+
methyl nitrosourea (MNU)[e]	1-80 mg/kg, i.p.	+
ethyl nitrosourea (ENU)[f]	10-250 mg/kg, i.p.	+
hycanthone[f]	150 mg/kg (free base), i.p.	+
triethylenemelamine (TEM)[f]	0.4-2 mg/kg, i.p.	+
X-rays[g]	200-1200 R	+
ethylene dibromide (EDB)[h]	50-250 mg/kg, i.p.	-
dimethyl nitrosamine (DMN)[f]	4-8 mg/kg, i.p.	-
diethyl nitrosamine (DEN)[f]	125 mg/kg, i.p.	-
benzo[a]pyrene (B[a]P)[f]	250-500 mg/kg, i.p.	-
caffeine[f]	200 mg/kg	-
N-methyl-N'-nitro-N'-nitrosoguanidine (MNNG)[f]	25-50 mg/kg, i.p.	-
mitomycin C[f]	4 mg/kg, i.p.	-

[a]The criterion for a positive UDS response was that the treated germ cells had to show at least 2 × the incorporation of [^{3}H]dThd as did the controls. For those agents giving a positive response, UDS was detected over the entire exposure range indicated.

[b]Sega et al., 1976.

[c]Sotomayor et al., 1978.

[d]Cumming and Michaud, 1979.

[e]Sega et al., 1981.

[f]G. A. Sega, unpublished data.

[g]Sega et al., 1978.

[h]Sega and Sotomayor, 1980.

Figure Legends

Figure 1. Timing of spermatogenesis and spermiogenesis in the mouse. The last scheduled DNA synthesis takes place in pre-leptotene primary spermatocytes. Mutagen treatment can induce UDS in germ-cell stages from leptotene through mid-spermatids. (Spermatid stages are labeled from S_1 to S_{16}.) No UDS can be detected in later stages.

Figure 2. Initial level of UDS (occurring within ~4 h after treatment) induced in early spermatids of (C3H × 101)F_1 mice as a function of i.p. exposure to MNU. Error bars are ±1 S.E.M. The least-squares linear fit curves are shown by a solid line for the 3-month old males (●) and a dashed line for the 1-year old males (○) [From Sega et. al., 1981].

Figure 3. Binding of [^{3}H]B[a]P and its metabolites to liver DNA (○--○) and testicular DNA (●—●) in (C3H × 101)F_1 hybrid mice as a function of time after i.p. exposure to 200 mg/kg of the chemical. The specific activity was 4.4×10^{-11} disintegrations per minute/molecule of B[a]P.

Figure 4. Alkylation level of testicular DNA of the mouse at various times after inhalation exposure to 3 ppm-h of ETO.

Figure 5. Absence of a UDS response in early spermatids of (C3H × 101)F_1 mice after various i.p. exposures to EDB.

Figure 6. Alkylation of DNA from liver, testis and sperm of (C3H × 101)F_1 mice as a function of time after exposure to ^{3}H-labeled EDB.

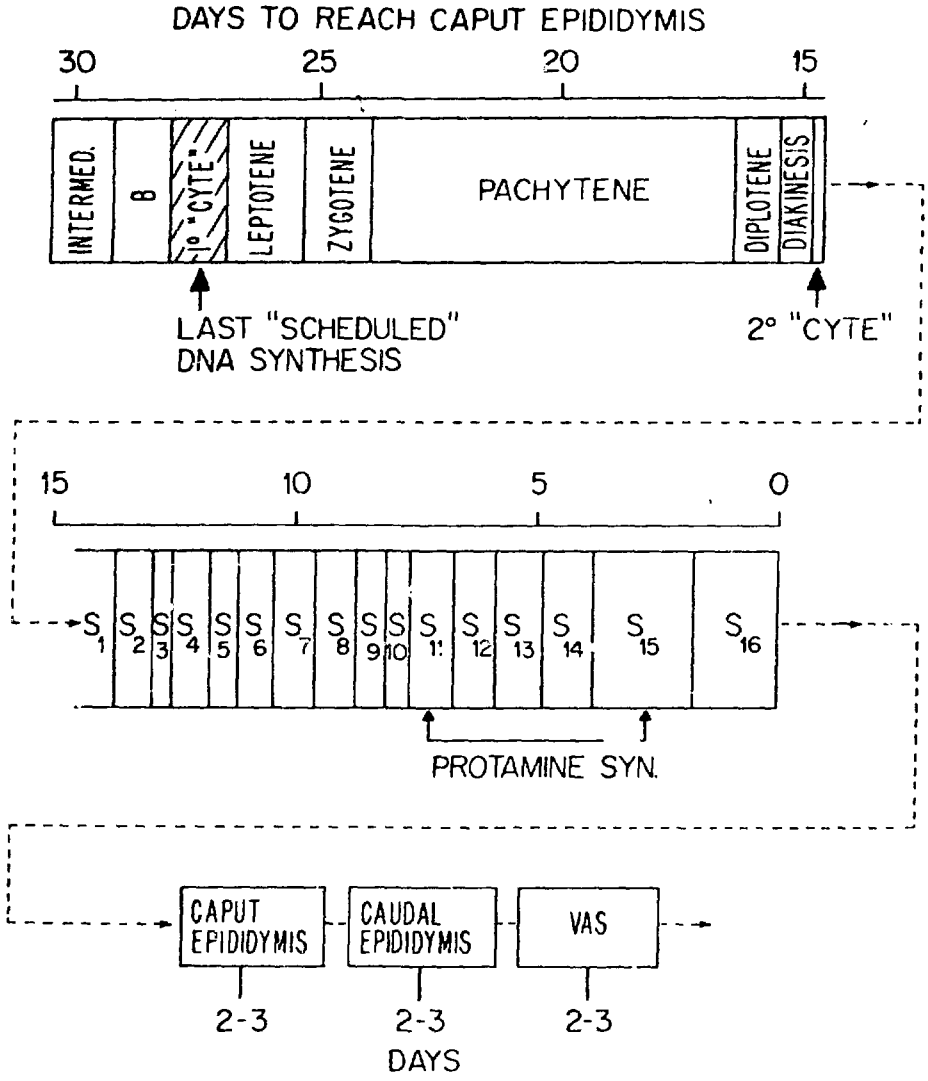
DAYS TO REACH CAPUT EPIDIDYMIS
30
25
20
15
INTERMED.
B
1° "CYTE"
LEPTOTENE
ZYGOTENE
PACHYTENE
DIPLOTENE
DIAKINESIS
LAST "SCHEDULED" DNA SYNTHESIS
2° "CYTE"
15
10
5
0
S1
S2
S3
S4
S5
S6
S7
S8
S9
S10
S11
S12
S13
S14
S15
S16
PROTAMINE SYN.
CAPUT EPIDIDYMIS
CAUDAL EPIDIDYMIS
VAS
2-3
2-3
2-3
DAYS

FIG. 1

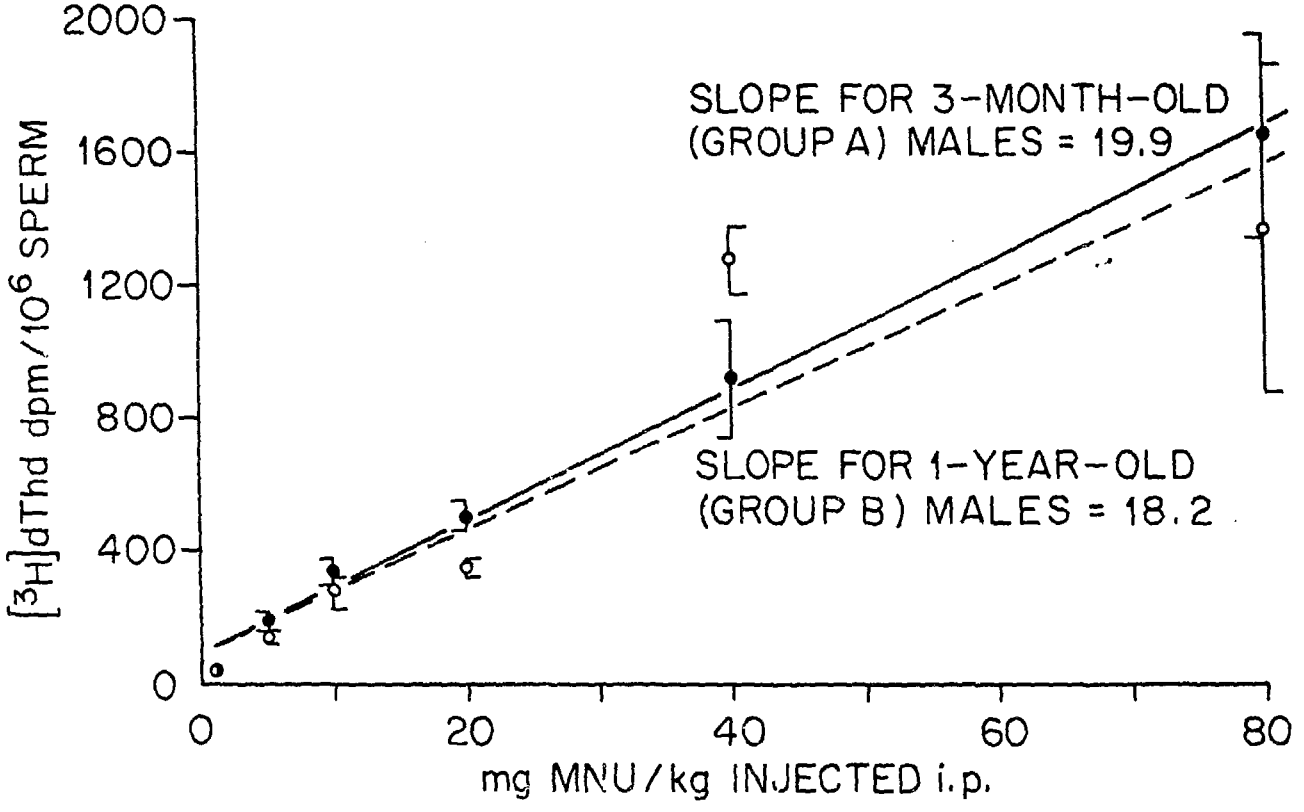
2000
1600
1200
800
400
0
[3H]dThd dpm/10^6 SPERM
0
20
40
60
80
mg MNU/kg INJECTED i.p.
SLOPE FOR 3-MONTH-OLD
(GROUP A) MALES = 19.9
SLOPE FOR 1-YEAR-OLD
(GROUP B) MALES = 18.2

FIG. 2

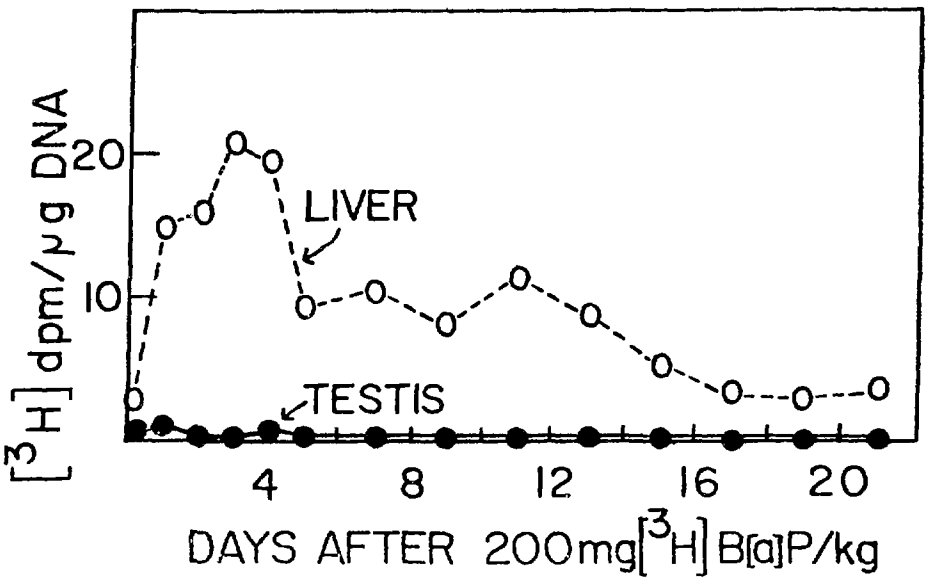
[3H] dpm/µg DNA
20
10
LIVER
TESTIS
4
8
12
16
20
DAYS AFTER 200mg[3H] B[a]P/kg

FIG. 3

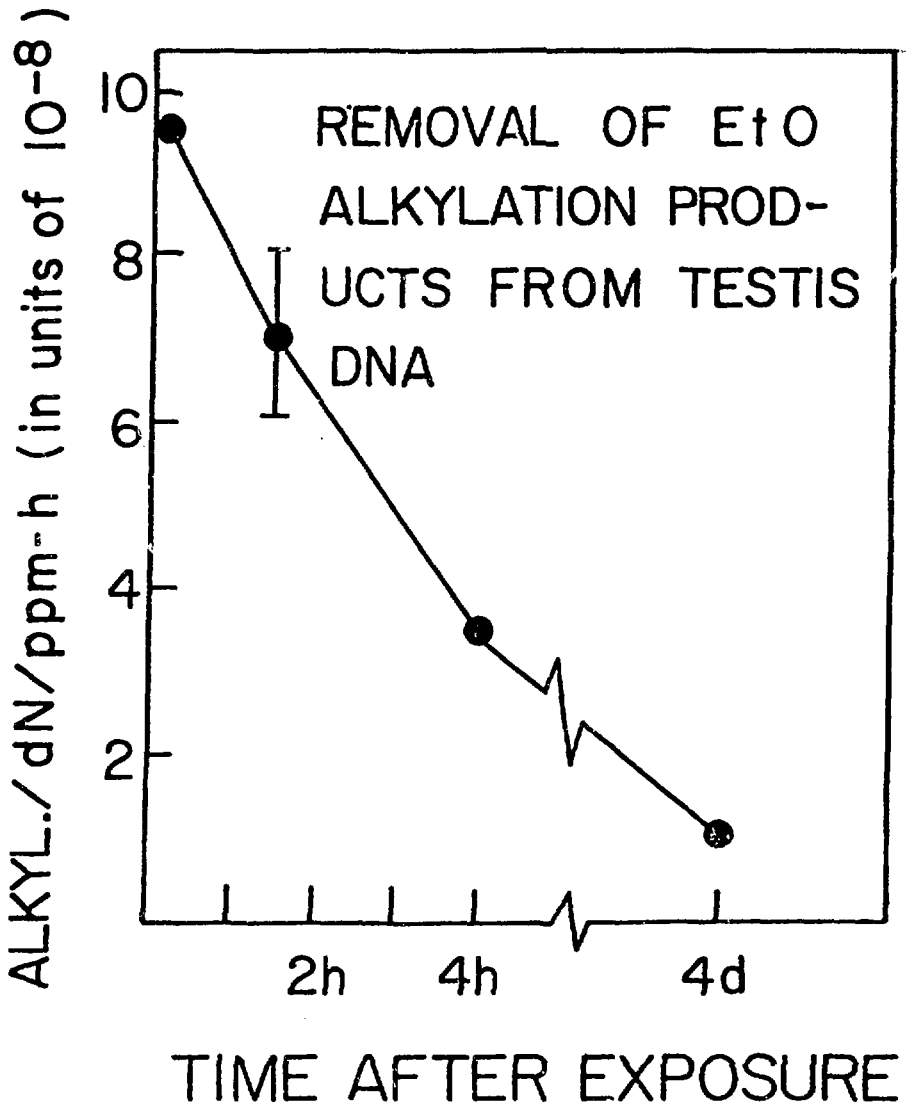
REMOVAL OF EtO ALKYLATION PRODUCTS FROM TESTIS DNA
ALKYL./dN/ppm-h (in units of 10-8)
10
8
6
4
2
2h
4h
4d
TIME AFTER EXPOSURE

FIG. 4

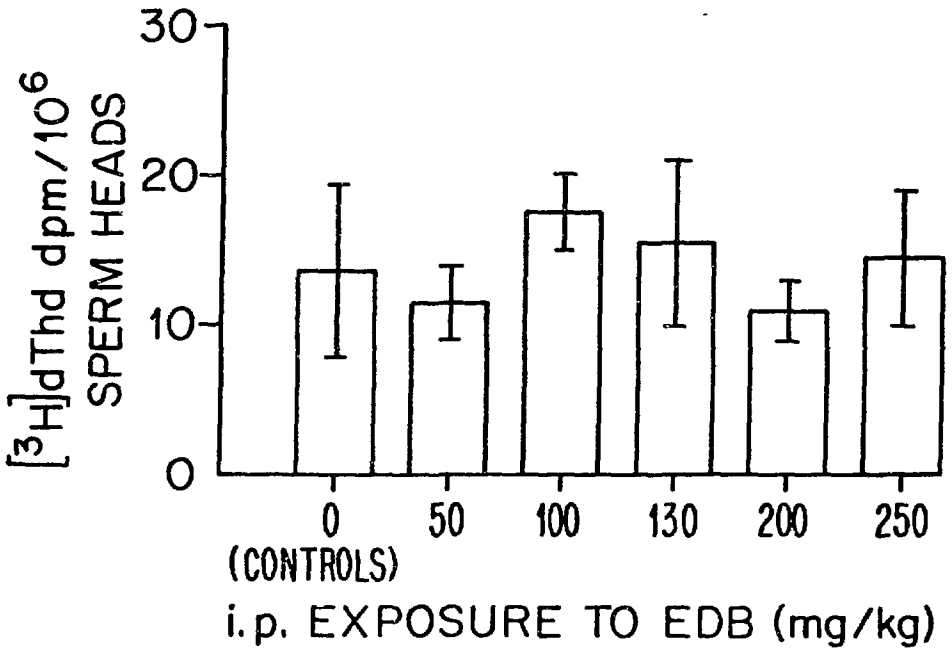
[3H]dThd dpm/10^6 SPERM HEADS
30
20
10
0
0 (CONTROLS)
50
100
130
200
250
i.p. EXPOSURE TO EDB (mg/kg)

FIG. 5

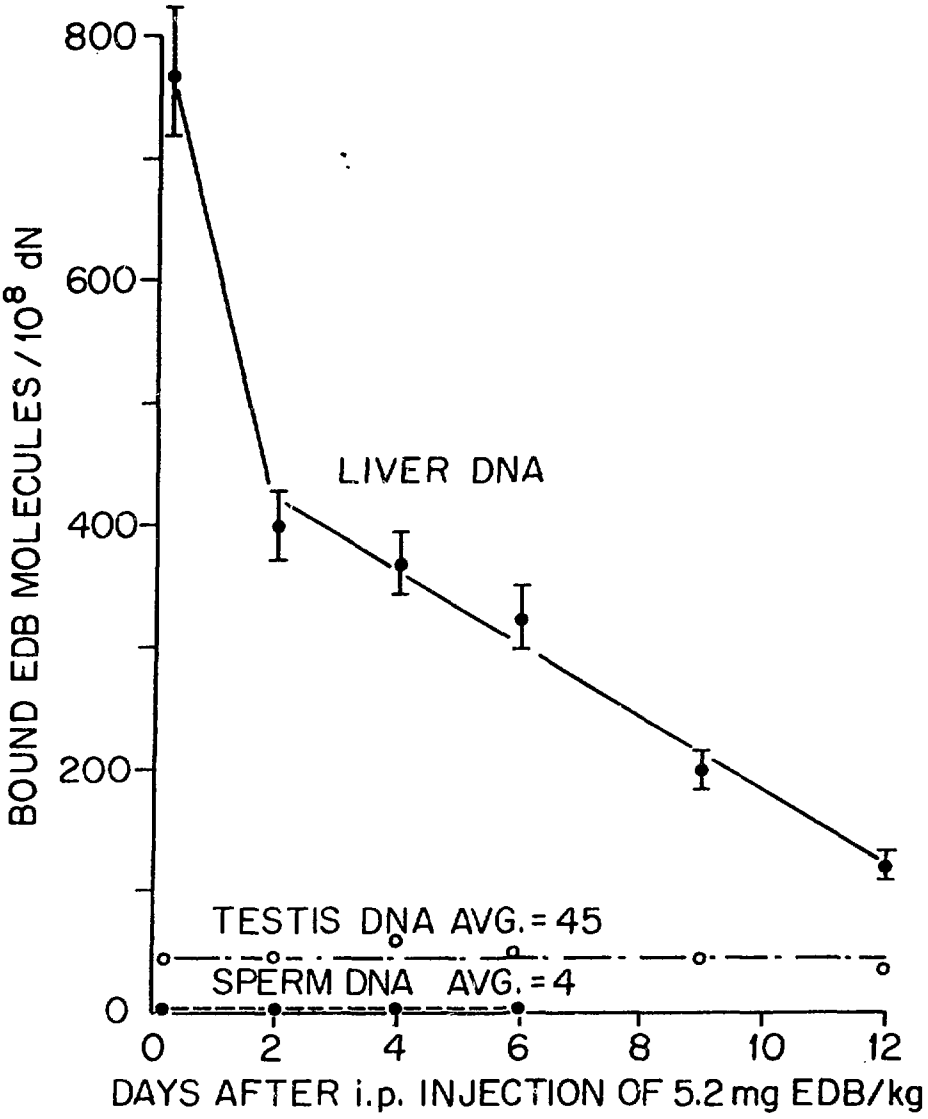
800
600
400
200
0
BOUND EDB MOLECULES / 10^8 dN
LIVER DNA
TESTIS DNA AVG. = 45
SPERM DNA AVG. = 4
0
2
4
6
8
10
12
DAYS AFTER i.p. INJECTION OF 5.2 mg EDB/kg

FIG. 6