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Frank Charles Janca

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STABILITY OF INDUCED DNA ALKYLATIONS IN SPERM
AND EMBRYOS OF DROSOPHILA MELANOGASTER.

THE LOUISIANA STATE UNIVERSITY AND
AGRICULTURAL AND MECHANICAL COL., PH.D., 1978

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STABILITY OF INDUCED
DNA ALKYLATIONS IN SPERM AND EMBRYOS
OF DROSOPHILA MELANOGASTER

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
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Doctor of Philosophy

in

The Department of Zoology

by
Frank Charles Janca
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ABSTRACT

Treatment of Drosophila melanogaster spermatozoa with the alkylating agent ethyl methanesulfonate (EMS) induces a high level of DNA ethylation, but induces a comparatively low frequency of sex-linked recessive lethal mutations. The induction of recessive mutations is germ cell stage specific with the highest frequencies being induced when late spermatids or mature spermatozoa are treated. A high frequency of these induced mutations are mosaic mutations which apparently are established during early embryonic cleavage. Storage of EMS treated sperm cells in the seminal receptacle for prolonged periods causes a time dependent shift in the mutation spectrum. This storage effect results in a time dependent increase in the frequency of those types of mutational events that are classed as chromosome aberrations. To understand these aspects of the mutational spectrum it is important to determine if alkylations induced in the DNA of sperm cells are retained throughout cleavage in the embryo. Retention of DNA alkylations was investigated by double labeling male *Drosophila* with ^{32}P and ^3H -EMS and by comparing the $^3\text{H}/^{32}\text{P}$ ratios of DNA extracted from labeled sperm sampled at various times following EMS treatment with DNA extracted from embryos sampled throughout cleavage.

The $^3\text{H}/^{32}\text{P}$ ratios from the sperm samples indicate that DNA alkylations are lost from sperm cells at a very slow rate and that this loss can be accounted for by the hydrolysis of alkylated bases (ethyl purines) from the ethylated DNA. Because of this slow loss many of the alkyl groups accumulated in the treated spermatozoa are retained until

fertilization.

Comparison of the $^3\text{H}/^{32}\text{P}$ ratios of sperm cells and embryos suggests that there is a significant reduction of alkylation during early embryonic development prior to the first or second mitotic division. This reduction during the early embryonic period cannot be accounted for by spontaneous hydrolytic loss of alkylated bases. After this early embryonic period alkyl groups continue to be lost but the rate of loss in these later embryonic stages is not significantly greater than the rate for hydrolytic depurination of alkylated DNA. Some alkyl groups are retained throughout early cleavage and are detected in the gastrula which is the latest embryonic stage sampled.

GENERAL SUMMARY OF ETHYL METHANESULFONATE MUTAGENESIS

Ethyl methanesulfonate (EMS) is a powerful mutagen for a wide variety of prokaryotic and eukaryotic organisms. EMS has been shown to induce various types of mutations, but the exact spectrum of mutations induced depends on the organism, tissue, and cell stage treated.

Prokaryotes

In bacteria and bacteriophage, EMS induces forward and reverse mutations (Loveless and Howarth, 1959; Strauss, 1961; Yanofsky et al., 1966) and apparently induces a high frequency of transitional type mutations (mutations involving base pair substitutions where a purine is exchanged for another purine or a pyrimidine is exchanged for another pyrimidine). Kreig (1963) induced EMS revertants in r11 T4 bacteriophage mutants. The mutants used in this investigation were produced by mutagenic agents which cause specific types of DNA changes. Based on the types of mutants reverted Kreig concluded that the majority of EMS induced mutations in phage result from transitions of G:C $\rightarrow$ A:T, whereas a smaller portion are due to A:T $\rightarrow$ G:C transitions. Kreig also observed that EMS reverts proflavin induced frameshift mutations (mutations which result from addition or deletion of a single base pair in the DNA), and concluded that in addition to inducing transitions, EMS could induce single base pair deletions which result in frameshift mutations. Tessman et al., (1964) treated host-range mutants of the single-stranded DNA phages S13 and ϕ X174 with EMS and observed that guanine sites underwent transitional type changes more

frequently than did adenine sites. These authors also observed that in the single-stranded DNA the pyrimidine sites seemed to be mutated about as frequently as the complementary purine sites. Yanofsky et al. (1966) studied amino acid replacement in EMS induced revertants of tryptophan synthetase mutant strains of Escherichia coli. Observing that the majority of reversions involved single amino acid replacement the authors concluded that these amino acid replacements were most likely the result of single-base pair changes of the transition types.

Fungi and Eukaryotic Plants

Results similar to those obtained with E. coli were observed when the fungus Neurospora crassa was treated with EMS. Malling and de Serres (1968) found among 76 mutants induced at the Adenine-3b locus, 58% were transition mutations involving either G:C→A:T or A:T→G:C base changes; 9% were frameshift mutations involving single-base pair insertions or deletions; and 7% were considered to have involved more extensive intragenic deletions.

EMS induced both single locus mutations and chromosome aberrations in higher plants. Rao and Natarajan (1963) reporting the results of EMS seed treatment in barley (Hordeum vulgare L.) found that seed treatment resulted in an increased frequency of cytologically detectable chromosome aberrations (chromosome breaks, dicentric chromosomes and chromatid exchanges) in both mitotic root tip cells and meiotic pollen mother cells. The resulting M₁ (first generation) plants had an increased frequency of chlorophyll deficient chimeras which resulted from the induction of somatic chlorophyll mutations and exhibited a reduction in pollen and seed fertility. The M₂ (second generation)

plants resulting from EMS established lines were observed to have increased frequencies of visible mutations.

Ficsor (1965) treated Zea mays pollen with an EMS-mineral oil suspension and observed the induction of specific locus endosperm mutations involving three closely linked phenotypically distinguishable genes. The majority of these mutations involved simultaneous loss of all three genetic markers; however, a few of the endosperm mutants did involve single-gene mutations. EMS pollen treatment also increased the frequency of dominant effects (slow growth and pollen sterility) in the F_1 plants. Based on the multiple loss of endosperm markers and the occurrence of dominant effects Ficsor concluded that in maize pollen EMS induces mostly chromosome aberrations, but may be capable of inducing some single-gene mutations. Amano and Smith (1965) treated both seeds and seedlings of Zea mays with aqueous solutions of EMS. They observed that the majority of mutants induced involved single locus mutations which could have resulted from either single-base pair substitutions, single-base pair deletions or intra-cistronic deletions of several base pairs. The apparent inconsistency between the results of Ficsor and those of Amano and Smith is probably due to the treatment of different tissue types. Ficsor treated pollen gametes while Amano and Smith treated somatic cells of the seed embryos and growing seedlings. Treatment of gametes (pollen) allows the detection of both chromosome aberrations and single locus mutations in the resulting endosperms and embryos. Induction of chromosome aberrations in somatic cells, however, can lead to immediate metabolic changes which affect cell viability and growth. As a result the treatment of somatic cells provides a very specific screen for genetic damage and allows only those mutations

which do not significantly affect cell functions to be transmitted to the next generation. Bhatia (1967) reports that dimethyl sulfoxide exerts a synergistic effect with EMS in the induction of albina (chlorophyll deficient) mutants in the herb Arabidopsis thaliana.

Drosophila

EMS treatment of male Drosophila melanogaster induces a high frequency of both visible and lethal recessive mutations, (Fahmy and Fahmy, 1957 and 1961; Alderson, 1965; Epler, 1966; Jenkins, 1967a and 1967b) but the increased mutation frequency is observed only in specific germ-cell stages. Fahmy and Fahmy (1957 and 1961) observed that three-day sequential matings (broods) of EMS-injected males produced elevated frequencies of visible and sex-linked recessive lethal mutations only in those broods which result from spermatozoa that were treated during the post-meiotic stages. Jenkins (1967a and 1967b) also reported that EMS-induced visible mutations are stage specific and that they occur with greatest frequency when post-meiotic cells (spermatid and spermatozoa) are treated.

Another aspect of the EMS mutational spectrum in Drosophila is that a high frequency of the mutations induced are mosaic mutations producing individuals which contain both mutant and non-mutant cells (Jenkins, 1967a and 1967b; Epler, 1966; Alderson, 1965; Lee et al., 1970). Jenkins (1967a) observed that in 405 F₁ EMS induced dumpy mutants 315 (78%) were visibly mosaic and 90 (22%) were visibly complete. Jenkins estimated that when correction was made for those mutants which were visibly complete but gonadally mosaic the frequency of F₁ mosaic mutants was approximately 98%. Lee et al. (1970) also observed a high frequency of EMS-induced mosaic yellow-body F₁ mutants (86%)

and mosaic white-eye mutants (49%). The observation that 51% of the white-eye mutants are completes is attributed to the finding that the nuclei of the eye imaginal disc represent only a small sample of the original cleavage nuclei. Thus, mutant individuals can be mosaic but if mutant nuclei are not included in the eye imaginal disc the mosaic is not detected as a visible mutation in the F_1 . Lee (1976) has combined data from several sources and estimates that among mutagen-induced visible-mosaic F_1 females 11% carry a mosaic germline; 13% carry a completely mutant germline and 76% carry a completely non-mutant germline. This indicates that on the average mosaic embryos carry an induced mutation in only 1/4 of their nuclei. Thus, as observed by Auerbach (1946) the fixation of these mosaic mutations does not occur immediately after treatment of the sperm cells but apparently is delayed until the first or second mitotic division in the embryo.

Storage of EMS treated sperm cells in the seminal receptacle of the female causes a time dependent change in the mutation spectrum (Abrahamson et al., 1969; Srám, 1970a and 1970b; Schalet, 1977). Abrahamson et al. (1969) reported an increase in translocation frequency after 10-day storage of EMS treated sperm. Only 20 F_1 cultures from the stored group were fertile and capable of being further tested for translocations. The number of translocations observed in the stored group (three translocations) is not much greater than the number observed in the non-stored group (one translocation) and thus the difference between the stored and non-stored groups may not be statistically significant. However, the sharp drop in the fertility among the stored cultures could indicate an EMS storage effect. Srám (1970a)

treated *Drosophila* males with EMS and compared the frequency of dominant lethals in seven sequential egg broods taken over a 12 day period.

The frequency of dominant lethals shows a continual increase with storage time. Schalet (1977) examined four sequential broods of progeny derived from a group of EMS treated sperm cells stored over a 12 day period and observed that later broods derived from stored EMS treated sperm cells had higher frequencies of sex-chromosome losses than did the earlier broods. Thus, the 'storage effect' appears to be a time dependent increase in the frequency of those types of mutational events (translocations, dominant lethals and sex-chromatin losses) that are classed as chromosome aberrations.

Aaron and Lee (1978) reported that exposure of *Drosophila* males to 25 mM EMS induced a dosage of 1.42×10^{-2} ethylations per nucleotide in the sperm cells. This dose is equivalent to ethylation of 1.4% of the total nucleotides in the sperm cell or ethylation of about 7% of the guanines (Lee, 1976) most probably at the N-7 position. Parallel genetic tests of males treated at the same high dosage indicated that only 43% of the treated X-chromosomes carried sex-linked recessive lethals (Aaron and Lee, 1978). Using data for dose and genetic response from treatments with high and low levels of exposure to ^3H -EMS these authors have constructed a dose-response curve. The relative frequency of sex-linked recessive lethals increases linearly with dosages ranging from 2.1×10^{-4} to 1.42×10^{-2} ethylations per nucleotide. This dose-response curve does not exhibit a dose threshold in relation to mutation induction and predicts that the dose of ethylation required to double the frequency of sex-linked recessive lethals is 4×10^{-5} ethylations per nucleotide. This is equivalent to requiring the ethylation of

approximately 12,000 nucleotides for each doubling of mutation rate. Thus, it appears that ethylation of sperm cell DNA is not very efficient in inducing lethal mutations.

Mammalian Systems

Several groups of investigators (Ehling et al., 1968; Cattanaach et al., 1968; Generoso and Russell, 1969; and Generoso et al., 1974) have reported that intraperitoneal (i.p.) injection of EMS induces dominant lethal mutations in mice. In male mice the induction of dominant lethal mutations is germ-cell stage specific. Cattanaach et al., (1968) found the greatest increases in dominant lethal frequencies among progeny of matings made 6-7 days after i.p. injection, where the sperm had been treated as mature spermatozoa in the epididymis and as late spermatids in the testes. Sega (1974) using radiolabeling techniques has been able to estimate more precisely the most sensitive stage for dominant lethal induction. His data indicated that the spermatozoa which gave the highest dominant lethal frequencies were mid-spermatids at the time of EMS treatment. Ehling et al., (1968) pointed out that dominant lethal mutations appeared to be induced most frequently in those stages which were undergoing nuclear histone exchange (exchanges of lysine-rich for arginine-rich histones) and suggested that the germ cell sensitivity was due to metabolic changes in these post-spermatogonial cells. Sega (1974) has shown that the mid spermatids do not exhibit the unscheduled DNA synthesis seen in earlier stages in response to EMS alkylation. This implies that spermatids may have lost an ability to repair DNA alkylation damage. Cattanaach et al., (1968) observed that the greatest frequency of translocations resulting from chromosome breakage after i.p. injection occurred during the period in which the greatest frequencies of dominant lethal mutations were induced, suggesting that dominant

lethal mutations are primarily due to chromosome breakage and the resulting rearrangement of the genetic material. Generoso et al. (1974) observed similar relations between stage of treatment and induction of dominant lethal mutations and translocations following i.p. injections of EMS above 100mg/kg body weight.

EMS has also been shown to produce mutations under in vitro conditions in mammalian tissue culture systems. Chu and Malling (1968) provided evidence that EMS-induced specific locus mutations in isolated Chinese hamster cell mutant clones. These clones were either auxotrophic for L-glutamine (glu⁻) or resistant to 8-azaguanine (azg^r). EMS significantly increased the reverse mutations at the glu locus and the forward and reverse mutations at the azg locus.

EMS Induced Alkylated DNA Products

Ethyl methanesulfonate belongs to the group of biologically active compounds designated alkylating agents. The biological effects of these chemical agents have been attributed to their ability to alkylate cellular macromolecules (protein and nucleic acids) under physiological conditions (Drake, 1970; Loveless, 1966; Lawley, 1974a and 1974b). EMS is a monofunctional alkylating agent which reacts biochemically to ethylate nucleophilic sites on the cellular macromolecules. The alkylating reaction between EMS and biological macromolecules has been described as a slightly modified bimolecular (S_N2) nucleophilic substitution (Lawley, 1974a; Osterman-Golkar, et al., 1970) and EMS has been designated an S_N1/S_N2 type alkylating agent. The types of products formed when EMS reacts with the nucleic acids (DNA and RNA) are of particular interest since it is assumed that these products lead to promu-

tagenic (events which require further modification to result in mutations) and mutagenic events (Lawley, 1974b; Strauss, et al., 1975; Verly, 1975). DNA has several sites which can react with EMS. Among these are the ring and amino nitrogens and keto oxygens of the bases and the oxygens of the phosphate groups which link the sugar components of the DNA backbone (Lawley and Brookes, 1963; Bannon and Verly, 1972; Sun and Singer, 1975).

Early investigations by Brookes and Lawley (1963) utilizing acid hydrolysis to separate ethylated DNA products indicated that under both in vitro and in vivo conditions the nitrogen positions were alkylated by EMS. Approximately 92% of the base ethylations occurred at the N-7 position of guanine and 8% occurred at the N-3 position of adenine. Based on these results it was presumed that alkylation of the N-7 position of guanine was the biologically significant event in relation to EMS induced mutagenesis (Lawley and Brookes, 1961; Lawley, 1966).

Loveless (1969) suggested that the oxygens on the bases, particularly the O-6 position of guanine, might also be alkylated in nucleic acids. Following this suggestion many investigators (Lawley and Thatcher, 1970; Lawley and Orr, 1970; Sun and Singer, 1975; Singer, 1976) found a significant proportion of total alkylations at the O-6 position of guanine when nucleic acids were treated under both in vivo and in vitro conditions with various S_N1 alkylating agents. S_N2 Alkylating agents do not readily react with the O-6 position of guanine possibly because it is less nucleophilic than other available sites (Sun and Singer, 1975; Lawley, 1974b).

Sun and Singer (1975) investigated the alkylation products resulting from treatment of HeLa cell DNA with EMS under in vivo and in

vitro conditions. Their methods involved a series of DNA degradation procedures. Utilizing a mild enzymatic digestion at neutral pH they were able to show that in addition to alkylating the nitrogen positions EMS also alkylated the O-6 position of guanine. The distribution of the ethylated DNA derivatives under their in vivo and in vitro conditions were similar. These investigators found that EMS ethylated nucleophilic base positions of DNA in the following order; 7-ethyl guanine (75-81%) > 3-ethyl adenine (2.2-4.4%) > 1-ethyl adenine; 7-ethyl adenine (2%) > 3-ethyl guanine; 3-ethyl cytosine; O⁶-ethyl guanine (less than 1%).

A substantial amount of the total alkylation (8-13%) was isolated as ethyl phosphate derivatives indicating that many DNA phosphodiester react with EMS to form deoxyribophosphotriesters. This is in agreement with observations made by other investigators (Lawley, 1974b; Bannon and Verly, 1972; Shooter, et al., 1974a and 1974b; Verly, 1974). Bannon and Verly (1972) reported that 15% of the total EMS alkylation in DNA is in the form of ethylated DNA phosphates. Shooter et al., (1974b) observed that 15% of the EMS-induced alkylations in the RNA phage R17 was also in the form of phosphotriesters.

Mutagenic Effects of Alkylated DNA Products

Since 7-alkyldeoxyguanosines ionize approximately 100 times faster than deoxyguanosine at pH 7.0, it was suggested that mutations might arise if the ionization of tautomeric shifts caused pairing errors during DNA replication (Lawley and Brookes, 1961). Such events would be expected to cause transition type mutations. Genetic evidence from prokaryotes tends to support this hypothesis for the induction of mutations (Kreig, 1963; Tessman et al., 1964). The majority of mutations induced by ethylation in prokaryotes are transitions and most of

the transitions involve base pair substitutions of G:C→A:T in the double stranded DNA (Kreig, 1963) and G→A in single-stranded DNA (Tessman et al., 1964).

Evidence from in vitro RNA polymerase systems using nucleotide templates that contain 7-methyl guanine indicates that the alkylated guanylic acid pairs normally with cytosine and that it mispaired no more frequently with uracil than does unmodified guanylic acid (Ludlum, 1970; Gerchman and Ludlum, 1973). Thus, alkylation of the N-7 position of guanine does not appear to effect its pairing properties and may not be the prime contributor of the transition mutations observed. Gerchman and Ludlum (1973) observed that use of polynucleotide templates containing O⁶-methyl guanylic acid results in misincorporation of UMP or AMP into the product co-polymer. Furthermore, the observation that cytosine (the normal pairing partner of guanine) does not block this misincorporation indicated that cytosine is not a normal base pairing partner for the O⁶-methyl guanylic acid and that O⁶-alkyl guanylic acid may not have the same pairing properties as guanylic acid. Thus, alkylation of DNA sites which are less reactive to alkylating agents may actually be more important for the induction of transition mutations than alkylation of the N-7 position of guanine.

While alkylation of the N-7 position of guanine may not be an important direct cause of mutations it may play a significant role by inducing promutagenic events which can be converted to mutagenic events. Lindahl and Nyberg (1972) have estimated that under normal physiological conditions (pH 7.5; 37°C) the rate constant for hydrolytic depurination of non-alkylated native DNA is $3 \times 10^{-11} \text{ sec}^{-1}$ ($1.8 \times 10^{-9} \text{ min}^{-1}$). Brookes and Lawley (1963) have calculated that the rate constants for

hydrolysis of N-7 methylated and N-7 ethylated deoxyguanylic acid in aqueous solutions are $7 \times 10^{-4} \text{min}^{-1}$ and $6 \times 10^{-4} \text{min}^{-1}$, respectively. Lawley (1975) estimated that in DNA at pH 7.0 the half-life for 7-methyl guanine is 150 hrs and the half-life for 3-methyl adenine is 30 hrs. Using these estimates Strauss et al., (1975) has calculated that the hydrolysis constants for loss of 7-methyl guanine and 3-methyl adenine from alkylated DNA are $7.7 \times 10^{-5} \text{min}^{-1}$ and $3.8 \times 10^{-4} \text{min}^{-1}$, respectively. Assuming the sites of the major reaction products 7-methyl guanine and 3-methyl adenine are the most frequently depurinated sites, Strauss et al., (1975) calculate that the overall rate constant for depurination is $1.84 \times 10^{-4} \text{min}^{-1}$. Since the ethylated reaction products are slightly more stable than the methylated products (Strauss and Hill, 1970) the hydrolysis rates for 7-ethyl guanine and 3-ethyl adenine and the over all depurination rate of these major EMS reaction products from DNA should be slightly slower than those for the methylated products. However, assuming their rate constants have the same order of magnitude as those of the methylated bases, the over all depurination rate for the ethylated products should be substantially larger than that calculated for non-alkylated native DNA.

A number of potentially mutagenic events could occur if apurinic DNA sites persist to the S-phase of the cell cycle. Apurinic sites might be ignored during DNA synthesis resulting in base pair deletions, and/or frameshift mutations. Alternately, the DNA synthesizing mechanism may select a base at random and insert it into the new DNA strand at the point opposite the apurinic site in the template strand (Fishbein et al., 1970). This event could lead to base pair substitution of either transition or transversion type. Depurination leaves an unstable

deoxyribose residue which can undergo spontaneous or enzymatic hydrolysis and result in single-strand breakage of the DNA chain (Lindahl and Anderson, 1972). In vivo DNA apurinic sites are converted to single-strand breaks by endonucleases which are specific for apurinic sites (Verly, 1975; Strauss et al., 1975; Kirtikar and Goldthwait, 1974). Such lesions if left unaltered could be converted to chromosome aberrations and/or dominant mutagenic events during the next round of replication (Margison and O'Conner, 1973; Bender, et al., 1974).

It has been proposed that formation of alkyl phosphotriesters through the reaction of nucleic acid phosphodiester and alkylating agents could have serious biological effects (Ross, 1958; Freeze, 1963). In nucleic acids phosphotriesters should be less stable than phosphodiester and should lead to hydrolysis of sugar-phosphate bonds introducing single-strand breaks into the nucleic acid structure. Bannon and Verly (1972) have shown that in bacteria, EMS and MMS have approximately the same toxicity; however, when EMS is reacted with DNA 15% of the total alkylations are alkylated DNA phosphates, while with MMS only 1% of the total alkylations occur on the phosphates. The same authors present evidence that phosphotriesters resulting from alkylation of DNA with EMS are very stable and remain intact after heating at 100°C for 90 min at pH 7.0. This evidence indicates that phosphotriesters formed in double-stranded DNA are stable and are probably not converted either enzymatically or chemically into single-strand breaks (Verly, 1974; Lawley, 1974a). Phosphotriesters formed in reactions with DNA probably do not contribute significantly to the mutagenic activity of EMS.

Shooter et al., (1971) have observed that RNA bacteriophages are

more sensitive to inactivation by monofunctional alkylating agents than are bacteriophages containing double-stranded DNA. Phosphotriesters of ribonucleotides readily hydrolyze at pH 7.0 at 37°C and this may account for the increased susceptibility of RNA phages to the monofunctional agents (Shooter, et al., 1971, 1974a and 1974b). RNA phosphotriesters can hydrolyze in one of two ways: 1) loss of the terminal part of the RNA molecule leaving a chain break or 2) simple loss of the alkyl group leaving the RNA chain intact (Shooter, et al., 1974b; Freeze, 1963). Thus formation of phosphotriesters in RNA phages may not in itself be a lethal or mutagenic event. Its biological effect may well depend on the manner in which it is hydrolyzed.

Enzymatic Recognition and Repair of Alkylated DNA

Strauss and Robins (1967) found that DNA methylated in vitro by the monofunctional alkylating agent MMS acts as a substrate for a specific nuclease activity from Micrococcus lysodeikticus crude extracts. This enzymatic activity produced single-strand breaks in the DNA strand which contained the methylated base and it had no effect on non-alkylated DNA. These authors suggest that perhaps two separate enzymes are active in the extracts. One enzyme recognizes and removes methylated bases while the second enzyme acts at apurinic sites to produce single-strand breaks.

Other investigators (Kirtikar and Goldthwait, 1974; Lawley and Orr, 1970; Margison and O'Connor, 1973; Lawley, 1973) also have provided evidence for the existence of intracellular enzymes which remove certain methylated bases and cause single-strand breaks at apurinic and possibly apyrimidinic sites. Lawley and Orr (1970) reported a rapid in vivo

removal of 3-methyl adenine and O⁶-methyl guanine from methylated DNA. The loss of these bases occurred faster than could be accounted for by chemical hydrolysis and the authors suggested that an excision type mechanism might account for this rapid removal. Margison and O'Connor (1973) working with in vivo rat systems found that alkylated DNA in rat liver cells lost 3-methyl adenine much more rapidly than purified alkylated DNA incubated in vitro at physiological pH. They also proposed that enzymatic excision might account for the rapid loss of this modified base.

Kirtikar and Goldthwait (1974) presented evidence that highly purified E. coli Endonuclease II in vitro was capable of breaking phosphodiester bonds in methylated DNA and releasing 3-methyl adenine and O⁶-methyl guanine. Verly and Rassart (1975) isolated a highly purified E. coli endonuclease which specifically breaks DNA at apurinic sites but does not act on methylated bases. Considering these findings Kirtikar and Goldthwait (1974) and Verly and Rassart (1975) suggested that Endonuclease II might consist of two enzymes, one for depurination and the second for breaking phosphodiester bonds.

Endonucleases which specifically recognize apurinic sites as substrate have been isolated from bacteria, plants and mammals (Verly and Rassart, 1975; Verly et al., 1973; Lindahl and Andersson, 1972; Ljungquist and Lindahl, 1974; Verly and Paquette, 1973). These enzymes cause single-strand breaks by hydrolyzing phosphodiester bonds near each apurinic site. Verly (1975) has shown that these enzymes are specific for DNA containing apurinic sites and that they do not hydrolyze intact DNA or DNA which contains only alkylated sites. Verly et al., (1974) have studied the effect of incubating labeled denatured depurinated T7

coliphage DNA for 3 hrs at 37°C with an E. coli endonuclease specific for apurinic sites, E. coli DNA polymerase I, T4 DNA ligase and a mixture of the 4 nucleoside triphosphates in Tris-Mg buffer. By the end of the incubation time both the number of apurinic sites and DNA breaks had decreased. Thus, in this in vitro system the apurinic sites were nearly all repaired. Omission of any of the three enzymes, but especially the endonuclease reduces the repair of the apurinic sites. Since an endonuclease for apurinic sites is observed in organisms of three kingdoms and can participate in an enzymatic in vitro repair of apurinic sites, these authors suggest that this endonuclease is a normal repair enzyme involved in maintenance of DNA structure in the cell. The fact that the endonuclease hydrolyzed the phosphodiester near apurinic sites and the fact that DNA polymerase I and DNA ligase are required for in vitro repair of apurinic sites suggests that repair could occur by means of an excision repair system. Certain excision repair systems are known to be error-prone (Witkin, 1975; Grossman, 1975) and if a repair process initiated by apurinic sites is error prone then increasing apurinic sites by alkylation of bases could indirectly lead to base pair error and increase the mutation rate.

Boyd and Presley (1974) studied repair replication of DNA in first instar larvae of Drosophila melanogaster using isopycnic centrifugation techniques. Larvae were pretreated with BUdR, FUdR, penicillin and Fungazone (BUdR labels the DNA, FUdR inhibits the activity of thymidylate synthetase, and Fungazone enhances the uptake of BUdR). The larvae were maintained on non-nutritive media to inhibit semi-conservative DNA replication and fed MMS or EMS in the presence of ³H-BUdR. Both MMS and EMS stimulated repair replication as indicated by an un-

scheduled non-semiconservative incorporation of ^3H -BUdR into DNA of the treated larvae. This repair replication was insensitive to caffeine and hydroxyurea both of which are inhibitors of semi-conservative synthesis. Whether this repair replication involves an endonuclease specific for apurinic sites or enzymes which identify alkylated bases has not yet been determined. However, cultured cells from certain MMS-sensitive mutant strains of *Drosophila* are also deficient for normal enzymatic UV-induced excision repair and apparently have a reduced ability to repair endonuclease sensitive sites (Boyd, et al., 1976; Boyd and Setlow, 1976). This suggests that some enzymes may recognize both alkylated and UV-altered DNA sites as substrates. Other MMS sensitive strains have been found to be deficient for a normal post-replication repair (Boyd, et al., 1975a and 1975b).

Sega (1974, 1976) observed that intratesticular injections of EMS with (250 mg/kg body weight) induced unscheduled DNA synthesis in various germ cell stages in male mice. This induced synthesis was detected by the unscheduled uptake of ^3H -dT into DNA and occurred in cell stages ranging from early to mid-meiotic prophase through early to mid-spermatid. EMS-induced unscheduled DNA synthesis was not observed in late spermatids. If the unscheduled DNA synthesis observed in early germ cell stages was the result of an enzymatic repair system, maturing spermatids, which undergo a number of physiological changes may lose the enzymes necessary for unscheduled synthesis and might not exhibit this ability. As spermatids mature arginine-rich replace lysine-rich histones in the nucleoprotein complex and the DNA becomes tightly coiled; perhaps the DNA of the late spermatid is too tightly coiled to be susceptible to enzymatic action, thus preventing unscheduled DNA synthesis.

Repair synthesis stimulated by treatment with alkylating agents has been reported in a number of different mammalian systems (Regan and Setlow, 1973; Roberts, et al., 1968 and 1971; Higgins et al., 1976; Ayad et al., 1969; Buhl and Regan, 1973; Scudiero, 1975). Roberts et al., (1968 and 1971) have observed that the level of repair replication in HeLa and Chinese hamster cells after treatment with MMS or EMS is directly proportional to the overall level of DNA alkylation. Comparing the results of experiments on loss of labeled methyl groups from alkylated DNA with those showing the absolute amount of 'repair synthesis' these authors estimate that for every alkyl group lost from DNA approximately 104 nucleotides are incorporated as a result of repair synthesis. Regan and Setlow (1973) have observed EMS-induced repair replication in tissue cultures of human skin fibroblasts and report that only two to three nucleotides are incorporated per lesion. Scudiero et al., (1975) have also observed that only a few nucleotides per lesion are incorporated by repair replication. Perhaps mammalian cells in general resemble bacterial cells which appear to have at least two types of excision repair systems (Witkin, 1975; Grossman, 1975). One type causes excision and replacement of long stretches of DNA while the second repairs by the replacement of much smaller regions of the DNA. Different mammalian cells could contain the information for several repair systems but preferentially use only one for repair.

A Statement of the Problem

The mutation spectrum induced by EMS in *Drosophila* has several characteristics which must be considered when attempting to understand the mutation mechanism. First, the induction of mutations is stage

specific with the highest frequency of induced mutations occurring when late spermatid and spermatozoa stages are treated (Fahmy and Fahmy, 1957 and 1961; Alderson, 1965; Epler, 1966; Jenkins, 1967a and 1967b). Second, exposure of males to 25 mM EMS induces a high frequency of alkylations in spermatozoa (1.4×10^{-2} alkylations per nucleotide or 1.4% of the nucleotides are alkylated), yet only 43% of the treated X-chromosomes have mutagen-induced sex-linked recessive lethal mutations (Aaron and Lee, 1978). Third, a high proportion of the mutations induced by EMS sperm treatment appear in mosaic progeny indicating that the fixation of these mutations does not occur immediately at the time of sperm treatment but is delayed until the first or second mitotic division in the embryo (Jenkins, 1967a and 1967b; Epler, 1966; Alderson, 1967; Lee *et al.*, 1970). Fourth, there appears to be a time dependent increase in the frequency of chromosome aberrations when EMS treated sperm cells are stored over a 12 day period (Abrahamson *et al.*, 1969; Srám, 1970a and 1970b; Schalet, 1977).

Work with many organisms has provided evidence for the existence of cellular enzymes which can recognize and remove alkylated bases in vivo leaving apurinic or apyrimidinic sites (Strauss and Robins, 1967; Kirtikar and Goldthwait, 1974; Lawley and Orr, 1970; Margison and O'Connor, 1973). Alkylated DNA bases are hydrolytically removed from treated DNA faster than are non-alkylated bases and the hydrolytic loss of bases from DNA also produces apurinic or apyrimidinic sites (Lawley and Brookes, 1963; Lindahl and Nyberg, 1973). A second type of enzyme (an endonuclease) which recognizes these apurinic or apyrimidinic sites and converts them to single-strand breaks has been isolated from bacteria, plants, and mammals (Verly and Rassart, 1975; Verly *et al.*, 1973; Lindahl

and Andersson, 1972; Ljungquist and Lindahl, 1974; Verly and Paquette, 1973). Endonucleases of this type have been shown to participate in in vitro repair of DNA carrying apurinic sites (Verly et al., 1974).

Methylation and ethylation of DNA stimulates the in vivo unscheduled DNA synthesis in *Drosophila* larvae mitotic cells (Boyd and Presley, 1974) and male mice germinal cells (Sega, 1974 and 1976). Unscheduled DNA synthesis in mice germinal cells occurs only in stages treated prior to the late spermatid and apparently does not occur in those cell stages which are most sensitive to induction of dominant lethal mutations (Sega, 1974). In *Drosophila* some strains which are EMS and MMS sensitive have also been found to be deficient for specific enzyme activities that are required for repair functions (Boyd and Setlow, 1975; Boyd et al., 1975).

It is possible that the various aspects of the EMS mutation spectrum in *Drosophila* described in the first paragraph of this section could result from the function or lack of function of a cellular mechanism capable of recognizing, removing and repairing ethylation damage of DNA. The stage specificity observed in *Drosophila* could result from the inability of a repair system to function during certain critical stages of spermiogenesis. Those cells which are most sensitive to EMS (late spermatids and spermatozoa) are undergoing or have undergone the morphological, physiological and metabolic changes which transform spermatids into spermatozoa (Cooper, 1965; Das et al., 1964a and 1964b; Olivieri and Olivieri, 1965; Brink, 1968). These changes could result in internal environmental changes which effect the functioning of repair enzymes or the loss of the enzymes themselves. Either situation could result in an accumulation of alkylations and/or alkylation damage on the sperm DNA. The observation that a high level of DNA alkylation on sperm cell

DNA is relatively inefficient in inducing sex-linked recessive lethals (Aaron and Lee, 1978) might also result from the action of a removal mechanism which functions after fertilization in the embryo to remove and repair alkylated DNA. The enzymes, if any, involved in this embryonic system could come from sperm or ovum. The fact that a large proportion of the EMS-induced mutations are mosaic in nature could also be accounted for by an embryonic repair system which functions during the first few mitotic divisions to remove alkylations from the embryonic DNA.

If Drosophila melanogaster has the capability of removing alkylated DNA products this removal ability should be detectable through use of appropriate radiolabeling techniques. The remainder of this dissertation describes and discusses the results of a series of double-radiolabeling experiments designed to investigate the existence of such a removal system in Drosophila melanogaster embryos and sperm cells.

METHODS AND MATERIALS

The sex chromosome complement of the normal *Drosophila* male and female genomes consists of a free X- and a free Y-chromosome, and two free X-chromosomes, respectively. Normally the X-chromosome consists of a long left arm (XL), a centromere and a minute right arm (XR). The normal Y-chromosome consists of a long arm (YL), a centromere and a short arm (YS) and carries two sets of factors necessary for male fertility. The first set of male fertility factors (KL) is on the YL and consists of five cistrons (k11-k15), while the second set (KS) is on the YS and consists of two fertility genes (ks1 and ks2). Loss of the entire Y or either of the Y arms or even any one of the male fertility genes results in male sterility. However, rearrangement of the Y-chromosome without loss of any part of it does not effect the fertility of males. The X- and Y-chromosomes each carry a normal allele for the production of ribosomal RNA. This locus located near the centromere of either chromosome is designated the bobbed locus (bb). Genomes that are deficient for the bobbed locus are genetically imbalanced and inviable (Lindsley and Grell, 1972).

Mating Scheme for the Males

The mating scheme for the production of the males used in these experiments is illustrated in Figure 1. Males of one stock (C) which carry a genetic rearrangement of sex-chromosomes are crossed to females of a second stock (D) that carry a different rearrangement of the sex-chromosomes. In stock C males the short arm of the Y-chromosome is

C males		X	D females	
<u>R(Y)L, bb⁻/X·Y^S, In(I)EN2, f bb⁺</u>			<u>y^L.scs¹, y⁺ac⁺bb⁺/ C(I)DX, y f bb⁻</u>	
Female gametes				
Male gametes	<u>y^L.scs¹, y⁺ac⁺bb⁺</u>		<u>C(I)DX, y f bb⁻</u>	
<u>R(Y)L, bb⁻</u>	<u>R(Y)L, bb⁻/</u> <u>y^L.scs¹, y⁺ac⁺bb⁺</u> (Nullo-X) INVIABLE		<u>R(Y)L, bb⁻/</u> <u>C(I)DX, y f bb⁻</u> (bobbed deficient) INVIABLE	
<u>X·Y^S, In(I)EN2, f bb⁺</u>	<u>X·Y^S, In(I)EN2, f bb⁺/</u> <u>y^L.scs¹, y⁺ac⁺bb⁺</u> (Balanced genome) viable fertile male		<u>X·Y^S, In(I)EN2, f bb⁺/</u> <u>C(I)DX, y f bb⁻</u> (Triplo-X) INVIABLE	

Figure 1. Mating scheme for the production of only fertile CD male embryos.

attached to the X-chromosome (X·Y^S) and the two ends of the long arm of the Y-chromosome are joined to form a circular Y-chromosome, the R(Y)L. The R(Y)L chromosome is deficient for the bobbed locus (bb⁻). The two X-chromosomes in the stock D females are joined at the centromeric ends to form a single tandem reversed acrocentric X-chromosome, (C(1)DX) which is deficient for the bobbed locus (bb⁻). To balance this deficiency the stock D genome contains a free Y^L·sc⁸¹ chromosome that carries a bobbed locus (bb⁺) and consists of a Y^L attached to a small piece of the X-chromosome.

Four embryonic genotypes are possible when stock C males are mated to stock D females (Figure 1). Because of the meiotic segregation of the rearranged sex-chromosomes in the parental flies only male embryos are genetically balanced and capable of producing viable fertile progeny. Two of the embryonic genotypes are inviable because they contain sex-chromosome imbalances (triplo-X and nullo-X embryos), while a third genotype is inviable because it is bobbed deficient (bb⁻).

Mating Scheme for the Females

The virgin females used in these experiments were produced according to the scheme illustrated in Figure 2. Stock F males which have a normal Y-chromosome and a complete but rearranged X-chromosome are mated to stock O females which have two complete but rearranged X-chromosomes each of which carries one sex-linked recessive lethal mutation (Lee, et al., 1972). The recessive lethals (1(1)J1 and 1) carried on the two X-chromosomes of the female are non-allelic and do not effect the viability of the stock O female.

Four embryonic genotypes are produced as a result of this cross

	F males	X	O females
	$\frac{\text{In(I) dl-49+B}^{M1}}{y \text{ ac v B}^{M1}} / Y$		$\frac{X \cdot Y^S, \text{In(I)dl-49 + sc}^{J1}}{\text{In(I)sc}^8, \text{sc}^8 \text{ l B}}$
	Female gametes		
Male gametes	$\frac{X \cdot Y^S, \text{In(I)dl-49 + sc}^{J1}}{\text{l(I)Jl } y^s \text{ v f B}}$		$\frac{\text{In(I)sc}^8, \text{sc}^8 \text{ l B}}$
Y	$\frac{Y/X \cdot Y^S, \text{In(I)dl + sc}^{J1}}{\text{l(I)Jl } y^s \text{ v f B}}$ Hemizygous lethal INVIALE		$\frac{Y/\text{In(I)sc}^8, \text{sc}^8 \text{ l B}}$ Hemizygous lethal INVIALE
$\frac{\text{In(I)dl-49 + B}^{M1}}{y \text{ ac v B}^{M1}}$	$\frac{\text{In(I)dl-49+B}^{M1}, y \text{ ac v B}^{M1}}{X \cdot Y^S, \text{In(I)dl-49 + sc}^{J1}, \text{l(I)Jl } y^s \text{ v f B}}$ VIALE VIRGIN		$\frac{\text{In(I)dl-49+B}^{M1}, y \text{ ac v B}^{M1}}{\text{In(I)sc}^8, \text{sc}^8 \text{ l B}}$ VIALE VIRGIN

Figure 2. Mating scheme for the production of Q virgins.

<u>Sterilizer male</u>		X	<u>Oregon-R virgin</u>
<u>R(Y)L/X.Y^S</u>			<u>X/X</u>
		Female gametes	
		<u>X</u>	
Male gametes			
<u>R(Y)L</u>		<u>R(Y)L/X</u> (Deficient for Y ^S) STERILE MALE	
<u>X.Y^S</u>		<u>X.Y^S/X</u> FERTILE FEMALE	

Figure 3. Mating scheme for the production of wild-type automatic virgins.

(Figure 2). The two male embryonic genotypes (X/Y) each receive a lethal bearing X-chromosome from the maternal genome and since they are hemizygous for the X-linked recessive lethal these embryos are inviable. The other two embryonic genotypes (X/X) are viable since the lethal on the maternal X is balanced with a normal allele carried on the paternal X. The viable embryos produce females and since there are no males these females are all virgin. The females produced by this cross are given the designation Q virgins. Both genotypes were used in the following experiments.

Wild type automatic-virgin females are produced in large numbers by crossing small numbers of normal Oregon-R (wild type) females to sterilizer (R(Y)L/X·Y^S) wild type males. As illustrated in Figure 3 all male progeny resulting from crosses of Oregon-R virgins to R(Y)L/X·Y^S males are sterile because they lack the Y^S portion of the Y-chromosome and its associated fertility factors. Therefore, the females produced from this cross are automatically virgin.

³²P Labeling

Stock D females that had been inseminated by stock C males were placed on ³²P-labeled media in 1-pint glass milk bottles and were allowed to deposit eggs over a four-hour period. Each glass bottle contained 50 ml of a corn-meal agar media plus approximately 300 μ Ci of ³²P. The ³²P was incorporated into the media in the form of carrier-free H₃³²PO₄ (New England Nuclear) and the final ³²P concentration of the media was approximately 6 μ Ci/ml. of media.

The CD larvae which hatched from these eggs were maintained on the ³²P-labeled media until they became third instars, at which time

the larvae were floated off the ^{32}P -media with a 30% sucrose solution and washed with room temperature water to remove any ^{32}P media which might have been carried over during the floating procedure. The washed larvae were placed on non-radioactive media and allowed to develop without further disturbance into adult male flies. In this way the DNA of the CD males is randomly labeled with ^{32}P so the radioisotope can be used as a marker nuclide to detect sperm-derived DNA in sperm and embryo DNA extracts.

The number of ^{32}P atoms per sperm DNA = the number of ^{32}P dpm per sperm cell/the decay constant for ^{32}P . The number of dpm per sperm cell estimated for each embryo sample = dpm ^{32}P in the DNA extract of the sample/the number of embryos per sample corrected for fertility (Appendix I and Table VIII). Since polyspermy is a rare event in Drosophila melanogaster (Hildreth and Lucchesi, 1963) the denominator estimates the number of labeled sperm cells which contributed ^{32}P to the sample. The mean number of ^{32}P atoms per sperm cell DNA estimated using the samples from all experiments (Appendix 11 and Table 1) is 1413. Since there are approximately 3×10^8 nucleotides per Drosophila sperm DNA (Laird, 1973) this level of ^{32}P labeling is equivalent to 4.7×10^{-6} ^{32}P atoms per nucleotide.

^3H -EMS Treatment of CD Males

Four-day old adult ^{32}P -labeled CD males were treated with a tritiated form of the alkylating agent ethyl methanesulfonate (^3H -EMS). The ^{32}P -labeled males were exposed to the ^3H -EMS by one of two methods: 1) a sucrose feeding method or 2) a vapor treatment method. Two forms of the ^3H -EMS were used in this series of experiments. Ethyl-2- ^3H -methanesulfonate ([1- ^3H]EMS) was obtained from New England Nuclear

(Lot #599-183) at a specific activity of 355 mCi/mM and was used in two of the feeding experiments. Ethyl-1-³H-methanesulfonate ([1-³H]EMS) was obtained from New England Nuclear (Lot #816-209) at a specific activity of 6100 mCi/mM and was used in one feeding experiment and in the three vapor treatment experiments. Radiochemical purity was verified by showing that the labeled compound co-chromatographed with unlabeled EMS in the gas chromatograph (Aaron and Lee, 1978). The tritium label in the ³H-EMS was verified as being only on the ethyl group by showing that the only labeled hydrolysis product co-chromatographed with ethanol in the gas chromatograph (Aaron and Lee, 1978). The tritium labeled ethyl group could then be detected in DNA extracts from sperm and embryos.

The ³H-EMS was stored in dry ether to prevent radioautolysis and prepared for feeding by the method of Aaron and Lee (1978). A modification of the feeding method of Lewis and Bacher (1968) using glass filter supports (Aaron et al., 1977) was used to expose ³²P-labeled CD males to the ³H-EMS. Pieces of glass filter (7cm x 6cm) which are contained in glass vials (9 cm x 2.5 cm) are impregnated with ³H-EMS dissolved in a 1% sucrose solution. Fifty ³²P-labeled males are placed in each feeding vial and allowed to imbibe the ³H-EMS solution for 24 hours. Three feeding experiments were conducted. In two of the experiments the males were exposed to 12.2 mM [2-³H]EMS (specific activity, 355 mCi/mM) per vial. In the third experiment the males were exposed to 0.38 mM [1-³H]EMS (specific activity, 6100 mCi/mM) per vial. Using the log-exposure-log-dose relationship given in Aaron and Lee (1978) an EMS exposure level of 0.38 mM is equivalent to a dose of 7.5×10^{-4} ethylations per nucleotide on the DNA.

The apparatus and method described by Aaron et al. (1977) (Appendix III) was used to expose large numbers of ^{32}P -labeled CD males (500-1000) to $[1-^3\text{H}]\text{EMS}$ vapor. ^{32}P -labeled males were placed in a 250 ml vacuum flask and exposed to EMS vapor from 0.2 mg $^3\text{H-EMS}$ (10 mCi). This level of exposure gives a 6.0% mutation frequency in genetic tests for sex-linked recessive lethals (Aaron et al., 1977). Using the sex-linked recessive lethal frequency as a 'secondary dosimeter' the mutation frequency can be converted to dose in terms of alkylation per nucleotide. The dose-response curve of Aaron and Lee (1978) indicates that a 6.0% sex-linked recessive lethal frequency is equivalent to a dose of 6.7×10^5 ethylations per sperm cell. Considering that there are approximately 3×10^8 nucleotides per sperm cell (Laird, 1973) this dose is equivalent to 2.2×10^{-3} ethylations per nucleotide.

Sperm and Egg Sampling

The $^3\text{H-EMS}$ treated males were placed on non-radioactive media and allowed a 24 hour recovery period. Following the recovery period the treated males were mated to virgin females in a ratio of one male to five females. After the three day mating period the inseminated females are separated from the treated males and are used for the collection of sperm and egg samples.

An inseminated female stores the sperm transferred from the male in her seminal receptacle. Samples of sperm cells are obtained by removing the receptacles from a random sample of the inseminated females. The receptacles are dissected from the females into 0.1 standard saline citrate (SSC) and immediately frozen over dry ice on a small (0.5 cm x 0.5 cm) piece of glass filter. The dissected receptacles are

stored in the cold (-25°C) until DNA extraction.

Egg samples are collected using specially designed egg collecting chambers. The egg collecting chambers consist of eight-ounce plastic disposable beer glasses, squares of black sheath cloth (6.5 cm x 6.5 cm) and three cm pieces of plastic tubing (1.3 cm O.D.; 1.0 cm I.D). The plastic glasses are modified by cutting off the tip end of the neck and the upper four cm of the vessel. A piece of black sheath cloth is stretched over the mouth of the modified plastic glass and secured in place with a small rubber band. The inseminated females are divided into groups of 250 to 500 flies and each group is placed in a separate collecting chamber. Two identical collecting chambers are used for each group. One chamber is used to hold the flies when they are not being used for egg collection, while the second chamber is used when the flies are forced to deposit eggs. The plastic tubing is used to connect the open neck end of the holding chamber with the open neck end on the collecting chamber. The females are then shaken into the collecting chamber and subjected to an anesthetizing dose of carbon dioxide gas. Under these conditions an egg held in the uterus is released from the vagina. As an egg passes through the uterus it is penetrated by a sperm cell and thus released from the vagina as a newly fertilized egg (Miller, 1965). Once the females have deposited the eggs they are shaken back to the holding chamber and maintained on media with yeast supplement. The time lapse between anesthetizing the females and removal of the females is approximately five minutes. Therefore, when the females are removed from the collecting chamber, the eggs deposited on the cloth will range in age from 0 to 5 minutes.

The female flies deposit the eggs on the black sheath cloth and the walls of the collecting chamber. The black sheath cloth is carefully removed from the chamber and spread over a glass funnel. The eggs along the chamber wall are then washed onto the cloth with room temperature water. The number of eggs in each sample are counted under a dissecting microscope and then the eggs are allowed to develop at 25° C to the desired embryonic stage. When the embryos in a sample reach the proper embryonic stage as determined by the timed schedule of Rabinowitz (1941), development is stopped by transferring the embryos to a square piece (4.0 cm x 4.0 cm) of polyethylene and freezing them in 0.1 x SSC on a block of dry ice. Prior to freezing, embryos from each embryonic stage sampled were selected at random and cytologically prepared to verify the embryonic stage and determine the percent of embryos retained from stage to stage. The remaining embryos were stored at -25°C until DNA extraction. During the DNA extraction embryo samples stopped at the same stage of development were pooled to form larger samples of 1000 to 3000 embryos.

The cytological samples were prepared in the following manner. Eggs were dechorionated by placing them in a 1% Chlorox solution and gently brushing the chorion with a small (number 000) brush. The dechorionated eggs were placed on microscope slides (4 eggs/slide) and squashed in the presence of aceto-orcein stain (Oster and Balaban, 1963) under a thin (No. 0) cover glass. The cover glass was sealed to the slide with a clear finger nail polish. The preparations were allowed to stain at least a week before they were cytologically examined.

DNA Extraction Procedure

High molecular weight DNA was extracted from the sperm and embryo

samples by the enzymatic-phenolic extraction procedure described by Aaron and Lee (1978). By measuring the removal of ^{14}C -arginine this extraction procedure has been shown to remove more than 99% of the protein associated with the DNA (Aaron and Lee, 1978). Blank samples of DNA from non-labeled embryos and sperm were extracted to provide a means to accurately determine the background counts for the DNA cocktails. The purity of the extracted resuspended DNA dissolved in 0.1 x SSC was determined by measuring its UV-absorbance spectra using a Beckman Model DB-GT spectrophotometer. The absorbance of the 1 ml DNA solutions was measured versus a blank of 0.1 x SSC at wave lengths between 240 and 300 nm.

The amounts of ^3H and ^{32}P in each DNA sample was determined by liquid scintillation counting in a Packard Tri-Carb model 3320. The instrument has three counting channels (channels A,B,C) and was calibrated to give maximum ^3H efficiency in the A channel and maximum ^{32}P efficiency in the C channel. The DNA extract of each sample was transferred to a counting vial. Ten ml of instagel counting cocktail (Packard) is added to each DNA sample and the contents of the vial were shaken to insure uniform dispersion of the extract. The cocktails were placed in the refrigerated scintillation counter and allowed to cool and stabilize for at least 24 hours. The radiolabeled DNA samples and corresponding DNA blank samples were counted in 100 minute increments until 10,000 counts were accumulated in both the tritium and phosphorus channels.

Counts per minute (cpm) were converted to disintegrations per minute (dpm) by correcting for background counts, spillover between

channels, machine counting efficiency, and isotopic decay using a computerized data analysis system (Aaron and Lee; 1975). Spillover between channels, and counting efficiency were determined by the internal standardization method. The phosphorus counts were standardized for decay and spillover by adding aliquots (3.0×10^{-5} mCi) of the labeled phosphoric acid solution originally used to make the labeled media. Since the radioactive phosphorus in the sampled DNA and the original phosphoric acid solution have decayed to the same extent, this phosphoric acid internal standard has the same proportion of ^{33}P to ^{32}P for decay and spillover correction as the DNA sample. The phosphorus counts were standardized for efficiency by adding aliquots (3.0×10^{-5} mCi) of a ^{32}P certified standard (New England Nuclear).

The tritium counts were standardized by adding aliquots (approximately 3.0×10^{-5} mCi) of a ^3H -hexadecane certified standard solution (Amersham-Searle).

The determination of true cps in a counting channel due to a particular isotope required an adjustment for spillover between channels; i.e., counting shifts from one isotope into a channel counting another isotope. The spillovers are computed using the equations:

$$1) \text{ Spill over } A \rightarrow C = \alpha^{3\text{H}} = \frac{^3\text{H counts in Channel C}}{^3\text{H counts in Channel A}}$$

$$2) \text{ Spill over } C \rightarrow A = \alpha^{32\text{P}} = \frac{^{32}\text{P counts in Channel A}}{^{32}\text{P counts in Channel C}}$$

The correction in each channel for spill over is made using the equations:

$$3) \text{ True } ^3\text{H counts} = (N_{^3\text{H}} - B_{^3\text{H}}) - \alpha^{32\text{P}}(N_{^{32}\text{P}} - B_{^{32}\text{P}})/1 - \alpha^{32\text{P}} \alpha^{^3\text{H}}$$

$$4) \text{ True } ^{32}\text{P counts} = (N_{^{32}\text{P}} - B_{^{32}\text{P}}) - \alpha^{^3\text{H}}(N_{^3\text{H}} - B_{^3\text{H}})/1 - \alpha^{32\text{P}} \alpha^{^3\text{H}}$$

Where; N = counts per minute and B = background counts per minute.

The true cpm due to a particular isotope are converted to dpm using the equation:

$$5) \text{ dpm} = \frac{\text{cpm}}{(\text{counting efficiency})(\text{isotope decay coefficient})}$$

The half-life for ^3H is 12.26 years. Since the decay coefficient for ^3H is 0.00015 day^{-1} during the course of an experiment (3 months) the ^3H decay was minimal and for correction purposes was zero. ^{32}P has a half-life of 14.22 days and a decay coefficient of 0.04873 day^{-1} .

Therefore a decay correction must be made when computing ^{32}P dpm because a considerable amount of ^{32}P is lost during the course of an experiment. The ^3H and ^{32}P counts in each sample were corrected for decay back to the day of ^3H -EMS treatment.

The analysis of the counts on a series of certified sealed standards (Packard Instruments) provided an independent determination for the consistency of the efficiency of counting and spillover ratios for the liquid scintillation counter. These sealed standards were counted along with the DNA samples from the initial counting of the samples to the completion of the internal standardizations. The sealed standard series consisted of a sealed blank; unquenched samples of ^3H , ^{14}C and ^{36}Cl ; and quenched samples of ^{14}C . Thus, the consistency of performance for the liquid scintillation counter could be checked over a wide range of energy levels.

Statistical Analysis Based on Experimental Variance

A two-way analysis of variance using the F-test was performed on the $^3\text{H}/^{32}\text{P}$ ratios derived from the DNA extractions. The ratios are compared using a randomized block design to test the null hypothesis that there is no significant difference in variance due to treatment effects. The treatments in each comparison consist of the sperm cells and one or several of the embryo stages. Experimental variance was determined as variation among the $^3\text{H}/^{32}\text{P}$ samples within replicates.

A t-test was used to analyze observed rates of reduction of ^3H -ethyl groups in the DNA extracts to determine if these rates were the same as the rate of spontaneous hydrolytic depurination of alkylated DNA.

Calculation of Standard Deviations for the $^3\text{H}/^{32}\text{P}$ Ratios based on Sampling Error

The standard deviations based on sampling error for the $^3\text{H}/^{32}\text{P}$ ratios were computed in a stepwise manner. First, standard deviations based on total counting time and total counts uncorrected for decay were determined for the ^3H and ^{32}P counts separately. The standard deviations for the ^3H and ^{32}P counts were computed using the formula:

$$\text{Standard Deviation} = \frac{\sqrt{n_s t_b^2 + n_b t_s^2}}{t_b t_s} \quad (1)$$

where; n_s = The total counts on the sample

n_b = The total counts on the corresponding blank

t_s = The total time the sample was counted

t_b = The total time the corresponding blank was counted.

This formula was derived from the standard formula for determining the standard deviation based on sample counting rates:

$$\text{Standard Deviation} = \sqrt{\frac{r_s}{t_s} + \frac{r_b}{t_b}} \quad (2)$$

where; r_s = The counting rate of the sample

r_b = The counting rate of the corresponding blank

t_s = The counting time of the sample

t_b = The counting time of the corresponding blank.

$$\text{Since: } r_s = \frac{\text{Gross counts of sample}}{t_s} = \frac{n_s}{t_s}$$

$$\text{and: } r_b = \frac{\text{Gross counts of blank}}{t_b} = \frac{n_b}{t_b}$$

when substitution for r_s and r_b is made, equation (2) can be rearranged to yield equation (1).

In the second step the relative standard deviations for the ^3H and ^{32}P total counts uncorrected for decay were computed by dividing the standard deviation based on counting time for a particular isotope by the counting rate for the isotope. The relative standard deviations for the $^3\text{H}/^{32}\text{P}$ ratio was computed by adding the relative standard deviations for the ^3H and ^{32}P counts. The standard deviation for the $^3\text{H}/^{32}\text{P}$ ratio is determined by multiplying the relative standard deviation for the ratio by the value of the ratio.

RESULTS

Stability of Alkyl Groups in the Embryo

Six experiments were conducted to determine the stability of ethyl groups in the *Drosophila* embryo. Embryo samples were collected from either wild-type automatic virgins or Q virgins that had been inseminated by the ^3H -EMS treated ^{32}P -labeled CD males. Embryos were sampled at the following stages: 1) premitotic (15 minutes after deposition); 2) first mitotic division (20 and 25 minutes after deposition); 3) second mitotic division (30 minutes after deposition) and 4) gastrulation following cellularization of the embryonic syncytium (180 minutes after deposition) (Rabinowitz, 1941). In each experiment the males are mated for three days sequentially to two groups of females and the samples from these matings are designated Mating 1 for group 1 females and Mating 2 for group 2 females. The $^3\text{H}/^{32}\text{P}$ ratios derived from each mating is labeled accordingly (Table 1).

Among the first five experiments, sperm samples and the 180 minute embryo (gastrula) samples are common to all experiments. Table II shows the F-test analysis of variance for the $^3\text{H}/^{32}\text{P}$ ratios derived from the sperm and 180-minute embryo samples. The analysis was done in a randomized block design where treatments were the sperm and the 180 minute embryos, and each experiment was considered as a separate block. Therefore, there were two treatments, five blocks, and 19 degrees of freedom for the analysis. The ANOVA indicates that there was a significant difference ($P < 0.025$) between the isotopic ratios from the sperm cells and

TABLE I. THE $^3\text{H}/^{32}\text{P}$ RATIOS FROM SPERM AND EMBRYO SAMPLES FROM SIX DIFFERENT EXPERIMENTS

	Sperm Cells	Embryos (15 min)	Embryos (20 min)	Embryos (25 min)	Embryos (30 min)	Embryos (180 min)	Embryos (3-12 hrs)	p_1^g	p_2^h
<u>Experiment 1^{b,e}</u>									
Mating 1	0.51 ^a (± 0.01)	---	---	---	---	0.22 (± 0.003)	---		
Mating 2	0.62 (± 0.02)	---	---	---	---	0.10 (± 0.003)	---	0.17	---
<u>Experiment 2^{b,e}</u>									
Mating 1	1.15 (± 0.07)	---	---	---	---	0.10 (± 0.003)	0.08 (± 0.003)		
Mating 2	0.39 (± 0.06)	---	---	---	---	0.10 (± 0.003)	0.09 (± 0.003)	0.13	0.97
<u>Experiment 3^{c,f}</u>									
Feeding 1	1.39	---	0.11	---	---	0.18	0.13		
Mating 1	(± 0.32)		(± 0.02)			(± 0.02)	(± 0.01)		
Feeding 2	1.27	---	0.16	---	---	0.17	0.22	<0.01	0.53
Mating 1	(± 0.17)		(± 0.006)			(± 0.01)	(± 0.02)		

TABLE I (Continued)

	Sperm Cells	Embryos (15 min)	Embryos (20 min)	Embryos (25 min)	Embryos (30 min)	Embryos (180 min)	Embryos (3-12 hrs)	p_1^g	p_2^h
<u>Experiment 4^{d,f}</u>									
Mating 1	5.57 ($\pm .43$)	1.94 ($\pm .09$)	---	1.85 ($\pm .08$)	---	1.13 ($\pm .04$)	---		
Mating 2	2.12 ($\pm .08$)	0.23 ($\pm .004$)	---	0.18 ($\pm .008$)	---	0.18 ($\pm .009$)	---	0.018	0.59
<u>Experiment 5^{d,f}</u>									
Mating 1	0.32 ($\pm .09$)	0.10 ($\pm .01$)	---	0.11 ($\pm .01$)	---	0.08 ($\pm .01$)	---		
Mating 2	0.30 ($\pm .03$)	---	---	0.03 ($\pm .01$)	---	0.04 ($\pm .01$)	---	<0.01	0.67
<u>Experiment 6^{d,f}</u>									
Day 1	0.94 ($\pm .03$)	0.53 ($\pm .05$)	---	---	0.53 ($\pm .04$)	0.41 ($\pm .03$)	---		
Day 2	0.51 ($\pm .03$)	0.18 ($\pm .02$)	---	---	0.18 ($\pm .02$)	0.17 ($\pm .02$)	---	<0.01	0.24

TABLE I (Continued)

	Sperm Cells	Embryos (15 min)	Embryos (20 min)	Embryos (25 min)	Embryos (30 min)	Embryos (180 min)	Embryos (3-12 hrs)	P ₁ ^g	P ₂ ^h
Day 3	0.40 (±.01)	0.14 (±.05)	---	---	0.09 (±.04)	0.10 (±.04)	---		

Legend

- a Standard deviation calculated from the relative standard deviations based on sampling error.
- b ³²P-labeled males fed with 12.2 mM[2-³H]EMS (355 mCi/mM specific activity).
- c ³²P-labeled males fed with 0.38 mM [1-³H]EMS (6100 mCi/mM specific activity).
- d ³²P-labeled males vapor treated with 0.2 mg [1-³H]EMS (6100 mCi/mM specific activity).
- e Egg samples collected without carbon dioxide gas.
- f Egg samples collected with carbon dioxide gas.
- g P₁ is the probability level for the null hypothesis that the ³H/³²P ratio in the sperm are equal to the ³H/³²P ratio in all embryo stages based on an F-test ANOVA.
- h P₂ is the probability level for the null hypothesis that the ³H/³²P ratio in all embryo stages are equal based on an F-test ANOVA.

TABLE II. ANALYSIS OF VARIANCE FOR $^3\text{H}/^{32}\text{P}$ RATIO IN SPERM CELLS AND 180-MINUTE EMBRYOS FROM EXPERIMENTS 1 TO 5.

(The treatments are $^3\text{H}/^{32}\text{P}$ in sperm cells versus the 180-minute embryos, while the blocks are the separate experiments.)

	Sperm Cells	Gastrula (180-Minute Embryos)
Experiment 1	0.5142 0.6245 $\frac{1.1387}{1.1387} = S_{11}$	0.2237 0.0959 $\frac{0.3196}{0.3196} = S_{12}$
Experiment 2	1.1470 0.3880 $\frac{1.5350}{1.5350} = S_{21}$	0.1046 0.1035 $\frac{0.2081}{0.2081} = S_{22}$
Experiment 3	1.3820 1.2758 $\frac{2.6578}{2.6578} = S_{31}$	0.1833 0.1661 $\frac{0.3494}{0.3494} = S_{32}$
Experiment 4	5.5702 2.1232 $\frac{7.6934}{7.6934} = S_{41}$	1.1293 0.1847 $\frac{1.3140}{1.3140} = S_{42}$
Experiment 5	0.3195 0.2971 $\frac{0.6166}{0.6166} = S_{51}$	0.0777 0.0353 $\frac{0.1130}{0.1130} = S_{52}$

ANOVA TABLE

Source	Degrees of Freedom	Sum of Squares	Mean Square	F-test	Probability
Total	19	30.1419			
Stage	1	6.3475	6.3475	9.4612	$P < .025$
Experi- ment	4	11.2554	2.6139	3.8967	$P < .05$
Experi- ment x stage	4	5.8300	1.4575	2.1724	NS
Error	10	6.7090	0.6709		

the 180-minute embryos. This difference was due to a reduction of the $^3\text{H}/^{32}\text{P}$ ratios in the embryo samples. The ANOVA also indicated a block (experiment) effect ($P < 0.05$), but this difference between experiments can be attributed to different levels of labeling resulting from the different methods of administering the EMS and the differences in the specific activities of the ^3H -EMS samples used in the experiments (Table 1). There was no significant experiment-treatment interaction indicating that all five experiments were consistent in showing a statistically significant decrease in the $^3\text{H}/^{32}\text{P}$ ratios between sperm and the 180-minute embryo stage.

In experiments three, four and five, embryos were sampled at earlier stages to determine when during embryogenesis the events which cause the reduction in the $^3\text{H}/^{32}\text{P}$ ratio occur. When the ratios of the DNA samples from experiments three through five were compared, there was no significant difference between early and late embryos, but there was a significant difference between the sperm and embryo samples (Table 1). This indicates that the events which caused the reduction in the $^3\text{H}/^{32}\text{P}$ ratio occurred early during embryogenesis, possibly as early or earlier than the first embryo stages sampled. The probability levels (P_1 and P_2) given in the last two columns of Table 1 are based on experimental error from the analysis of variance and are a more conservative estimate of the difference between sperm cells and embryos than the confidence limits of the $^3\text{H}/^{32}\text{P}$ ratios in Table 1 which are based only on sampling error of the counting statistics.

A group of inseminated females from each mating in experiment five were set aside to investigate the effect of sperm storage on the $^3\text{H}/^{32}\text{P}$ ratio in the sperm. The sperm cells in these females were sampled

immediately after mating, 3 days after mating and 7 days after mating. Comparison of the ratios from the DNA extracts of the stored and non-stored spermatozoa suggested there was a time dependent decrease of ^3H -labeled ethyl groups in the stored sperm (Table IV).

Experiment six was designed to test both the stage effect (sperm vs. embryos) and the apparent storage effect observed in the stored sperm of experiment five. One thousand labeled CD males were treated with ^3H -EMS vapor and mated to 3400 virgin Q females. Nine hundred of these females were selected at random and set aside for sperm sampling. Sperm samples from the inseminated females were collected on each of the 3 days during which the embryos were collected. Embryo samples were pooled each day according to embryonic stage. The comparison of the $^3\text{H}/^{32}\text{P}$ ratios of the sperm DNA to embryo DNA was done using a randomized block design where treatments consisted of sperm cells, premitotic embryos (15-minute samples), second division embryos (30-minute samples) and gastrula embryos (180-minute samples). The samples were blocked according to the day (Day 1, Day 2, or Day 3) on which they were taken (Table III). Therefore, the variance can be partitioned into the component due to treatment differences (sperm vs. embryo stages) and the component, if any, due to storage differences.

The analysis of variance for experiment six showed a significant treatment effect ($p < 0.01$) and significant block (storage) effect ($p < 0.01$). The treatment effect was due entirely to the difference of $^3\text{H}/^{32}\text{P}$ ratios between the sperm cells and the embryos. This result was consistent with the results of the previous experiments. The difference among embryo samples was not significant; however, the sperm cells had a significantly larger ratio than any embryo sample. This indicates

TABLE III. THE ANALYSIS OF VARIANCE FOR $^3\text{H}/^{32}\text{P}$ RATIOS FROM EXPERIMENT 6.

	Sperm Cells	Premittotic Embryos (15 min)	2nd division cleavage Embryos (30 min)	Gastrula Embryos (180 min)
Day 1	0.9418	0.5292	0.3524	0.4053
Day 2	0.5052	0.1795	0.1768	0.1745
Day 3	0.4037	0.1375	0.0895	0.1034

ANOVA TABLE

Source	Degrees of Freedom	Sum of Squares	Mean Squares	F-test	Proba- bility
Total	11	0.7094			
Day	2	0.3984	0.1992	68.3	<.01
Stage	3	0.2935	0.0978	33.5	<.01
Sperm vs. Embryos	1	0.2887	0.2887	99.6	<.01
Gastrula vs. Earlier Stages	1	0.0043	0.0043	1.5	NS
Premittotic vs. 2nd Division Embryos	1	0.0004	0.0004	0.2	NS
Error	6	0.0175	0.0029		

TABLE IV. THE $^3\text{H}/^{32}\text{P}$ RATIOS FROM STORED AND NON-STORED
 ^3H -EMS TREATED DROSOPHILA SPERMATOZOA

	Mating	Day 1 (Non-stored)	Day 2	Day 3	Day 7
Experiment 5	1	0.3195 ^a ($\pm$.09)	---	0.1499 ($\pm$.02)	0.0583 ($\pm$.01)
	2	0.2971 ($\pm$.03)	---	0.1573 ($\pm$.04)	0.10 ($\pm$.01)
Experiment 6	1	0.9418 ($\pm$.09)	0.5052 ($\pm$.03)	0.4037 ($\pm$.02)	---
	2	0.7191 ($\pm$.04)	0.6638 ($\pm$.03)	0.6462 ($\pm$.03)	---

^aStandard deviation calculated from the relative standard deviations based on sampling error.

that the change in the $^3\text{H}/^{32}\text{P}$ ratio must have occurred earlier than 15 minutes after deposition of the egg. The storage effect was due to the decrease in the $^3\text{H}/^{32}\text{P}$ ratios of both sperm and embryos sampled on consecutively later days. This storage effect might be attributed to a time dependent loss of ethyl groups from the DNA of sperm cells stored in the seminal receptacle.

Stability of Alkyl Groups in the Sperm

Table IV shows the $^3\text{H}/^{32}\text{P}$ ratios of DNA extracts from the stored and non-stored sperm samples from experiments five and six. A comparison of the $^3\text{H}/^{32}\text{P}$ ratios from these four sperm samples indicates that there is a consistent time dependent reduction in the isotopic ratio with sperm cell storage.

Percentage of ^3H Retained in the Stored Sperm and Embryos

If the $^3\text{H}/^{32}\text{P}$ ratio of the non-stored sperm samples are considered unity, then the ratios from the stored sperm samples can be expressed as a percentage of the non-stored sample using the formula:

$$^3\text{H}\% = \frac{^3\text{H}/^{32}\text{P} \text{ stored samples}}{^3\text{H}/^{32}\text{P} \text{ non-stored samples}} \times 100$$

In a similar manner the $^3\text{H}/^{32}\text{P}$ ratios of the embryo samples can be expressed as a percent of the sperm samples. The loss of ^{32}P in the sperm cells and embryos is due to radioactive decay and since a correction is made for the decay in computing ^{32}P dpm, the ^{32}P terms in the numerator and denominator should cancel and the percentage obtained will approximate the percent of tritiated ethyl groups retained in the stored sperm

and the embryo samples.

One mechanism which accounts for loss of alkyl groups in DNA is excision repair (Regan and Setlow, 1973; Roberts et al., 1968 and 1971). If alkylated bases are removed by an excision repair, some of the ^{32}P atoms in the DNA backbone may also be lost. Regan and Setlow (1973) estimate that repair due to EMS damage involves the replacement of two to three nucleotides. The sperm derived DNA is randomly labeled with ^{32}P and the level of ^{32}P labeling on the sperm DNA is approximately 4.7×10^{-6} ^{32}P atoms per nucleotide. If the selective recognition and elimination of an ethyl group involves replacement of three bases the highest rate of removal of $^{32}\text{P} = 3(4.7 \times 10^{-6}) = 1.4 \times 10^{-5}$. Even if the excision of an ethylated base involves removal of 104 nucleotides as estimated by Roberts et al. (1968 and 1971), the highest rate of loss of ^{32}P is only 4.9×10^{-4} . Therefore, a decrease in the $^3\text{H}/^{32}\text{P}$ ratio indicates a selective removal of ethyl groups which exceeds the loss of the randomly labeled ^{32}P . Such a decrease would be effected by excision repair but would not be effected by changes in the efficiency of DNA extraction.

The majority of EMS induced alkylations on DNA occur at the N-7 position of guanine and N-3 position of adenine (Sun and Singer, 1975; Lawley and Brookes, 1963). It has been shown that more than 50% of the ethyl groups on the bases of ethylated sperm cell DNA co-chromatograph with 7-ethyl guanine (Aaron et al., 1973). Therefore, it is assumed that the majority of ^3H labeling of alkylated sperm DNA occurs at these sites. Approximately 15% of the ethylations are expected to occur on the DNA phosphates (Bannon and Verly, 1972), however, the phosphotriesters formed by alkylation of the DNA phosphates are stable and are not ex-

pected to contribute significantly to a reduction in the alkylation of ethylated DNA.

Strauss et al., (1975) has calculated the overall rate constant for hydrolytic depurination of 7-alkyl guanine and 3-alkyl adenine from alkylated DNA. This constant is based on the methylated DNA products which are slightly less stable than the ethylated products (Strauss and Hill, 1970). Assuming that this constant ($1.84 \times 10^{-4} \text{min}^{-1}$) also approximates the hydrolytic loss of the ethylated products; the observed reduction of ethylation in the stored sperm cells and in the embryos can be compared to the reduction expected from hydrolysis of alkylated purines.

The semi-log plot for ^3H -retained by sperm cells over the storage period is illustrated in Figure 4. All four series of sperm samples show at least a 10% decrease of ^3H label during the storage period. Rate constants (Table V) based on the observed decrease were computed by regressing the $\text{Log}_e N/N_0$ (dependent variable) with the negative time of storage (independent variable) such that;

$$k = \frac{\text{Log}_e N/N_0}{-\text{time}} = b_0 = \text{the slope of the regression,}$$

where: k = rate constant calculated

n = ^3H % after storage

n_0 = ^3H % before storage

t = time elapsed in minutes.

The observed rates of reduction of ethyl groups in the stored sperm do not differ greatly from the rate expected if this loss is due to spontaneous hydrolysis of alkylpurines from DNA.

Figure 4. The Reduction of ^3H -Label After Sperm Storage. Experiment 5 Mating 1 ▲ ; Experiment 5 Mating 2 ■ ; Experiment 6 Mating 1 ● ; Experiment 6 Mating 2 ● ; Hydrolytic depurination of alkyl purines ---.

TABLE V. THE CALCULATED RATES FOR $^3\text{H}/^{32}\text{P}$ REDUCTION IN STORED SPERM CELLS

Experiment	Time (minutes)	$\frac{{}^3\text{H}/{}^{32}\text{P} \text{ Sperm Stored}}{{}^3\text{H}/{}^{32}\text{P} \text{ Sperm Non-stored}} = \text{N}/\text{N}_0$	$\text{Log}_e \text{N}/\text{N}_0$	$k = \frac{\text{Log}_e \text{N}/\text{N}_0^a}{-\text{time}}$	
5	Mate 1	0	1.00	0.00	$1.66 \times 10^{-4} \text{min}^{-1}$
		4320	0.47	-0.75	(S.E. $2.56 \times 10^{-6} \text{min}^{-1}$)
		10080	0.19	-1.66	
	Mate 2	0	1.00	0.00	$1.16 \times 10^{-4} \text{min}^{-1}$
		4320	0.53	-0.63	(S.E. $9.47 \times 10^{-6} \text{min}^{-1}$)
		10080	0.33	-1.11	
6	Mate 1	0	1.00	0.00	
		1440	0.54	-0.62	$3.20 \times 10^{-4} \text{min}^{-1}$
		2880	0.43	-0.84	(S.E. $3.81 \times 10^{-5} \text{min}^{-1}$)
	Mate 2	0	1.00	0.00	
		4320	0.92	-0.08	$4.08 \times 10^{-5} \text{min}^{-1}$
		2880	0.90	-0.11	(S.E. $6.03 \times 10^{-6} \text{min}^{-1}$)

Mean $k = 1.61 \times 10^{-4} \text{min}^{-1}$ Pooled Standard Error = $2.62 \times 10^{-9} \text{min}^{-1}$

^aThe k value is computed from the regression of three points using a no intercept model for linear regression ($Y = kX$).

Figure 5 is the semi-log plots of ^3H %-retained by sperm derived DNA of the embryos from Mating 1 of each of the last three experiments. The data from the last three experiments were plotted since only these experiments contain data from three different measurements of the $^3\text{H}/^{32}\text{P}$ ratios of closely timed embryos (5-minute age span), and at least three points are necessary for performing a valid regression analysis for the effect of embryonic stage on the level of ^3H label (Table VI). Experiment three has three embryo data points (Table I) but the last point (3 hours to 12 hours) is from a collection of embryos with a 9-hour age span. Since only two of the samples in experiment three have the same age span in the embryo samples (5-minute age span) the data from experiment three was excluded from this regression analysis. To determine the most conservative estimate of the embryonic decrease in the ^3H label only the data for Mating 1 of experiments four and five and Day 1 from experiment six (Table I) were plotted since these experimental blocks exhibit the smallest embryonic decrease in ^3H labeling. Each point on the X-axis represents the minimum age of the embryo sample. As many as 21% of the embryos in a pooled embryo sample (Table IX) may have been retained for some period of time in the uterus of the female and were in more advanced stages of development than the majority of embryos in the sample. The retained embryos are of particular importance in the early embryo stages (15 to 30 minutes) since the time interval between stages is small. Therefore, the early embryo stage samples may overlap each other and may not cleanly separate the embryos into the discrete stages indicated previously. The regression lines for experiments four, five, and six, calculated from the three embryo points in each experiment, intercept the Y-axis at 36%, 34%,

Figure 5. The reduction of ^3H -label from sperm derived DNA during embryonic development. Experiment 4 Mating 1 $\blacktriangle$; Experiment 5 Mating 1 $\blacksquare$; Experiment 6 Mating 1 $\bullet$; Hydrolytic depurination of alkyl purines _____.

TABLE VI. THE CALCULATED RATES FOR $^3\text{H}/^{32}\text{P}$ REDUCTION IN THE EMBRYOS
DURING EARLY CLEAVAGE AND T-TEST OF THE RATES

Experiment	Time (minutes)	$\frac{{}^3\text{H}/{}^{32}\text{P Embryo}}{{}^3\text{H}/{}^{32}\text{P Sperm}} = \text{N}/\text{N}_0$	$\text{Log}_e \text{N}/\text{N}_0$	$\frac{\text{Log}_e \text{N}/\text{N}_0}{-\text{time}}$	
3	Mate 1	0 - 20	0.08	-2.5	$1.25 \times 10^{-1} \text{min}^{-1}$
	Mate 2	0 - 20	0.13	-2.0	$1.02 \times 10^{-1} \text{min}^{-1}$
4	Mate 1	0 - 15	0.35	-1.1	$7.00 \times 10^{-2} \text{min}^{-1}$
	Mate 2	0 - 15	0.11	-2.2	$1.47 \times 10^{-1} \text{min}^{-1}$
5	Mate 1	0 - 15	0.31	-0.9	$5.78 \times 10^{-2} \text{min}^{-1}$
	Mate 2	0 - 25	0.11	-2.2	$8.80 \times 10^{-2} \text{min}^{-1}$
6	Mate 1 Day 1	0 - 15	0.56	-0.6	$3.87 \times 10^{-2} \text{min}^{-1}$
	Mate 1 Day 2	0 - 15	0.36	-1.0	$6.81 \times 10^{-2} \text{min}^{-1}$
	Mate 1 Day 3	0 - 15	0.34	-1.1	$7.20 \times 10^{-2} \text{min}^{-1}$

TABLE VI (Continued)

Observed rates	Mean	Standard Error	Degrees of Freedom
1.25 x 10 ⁻¹ min ⁻¹			
1.02 x 10 ⁻¹ min ⁻¹			
7.00 x 10 ⁻² min ⁻¹			
1.47 x 10 ⁻¹ min ⁻¹	8.54 x 10 ⁻² min ⁻¹	1.14 x 10 ⁻² min ⁻¹	8
5.78 x 10 ⁻² min ⁻¹			
8.80 x 10 ⁻² min ⁻¹			
3.87 x 10 ⁻² min ⁻¹			
6.81 x 10 ⁻² min ⁻¹			
7.20 x 10 ⁻² min ⁻¹			

$$H_0: \mu_0 = 1.84 \times 10^{-4} \text{min}^{-1} = \mu$$

$$H_a: \mu \neq 1.84 \times 10^{-4} \text{min}^{-1}$$

with: $\alpha = 0.05$

Limits are: $-2.306 \leq t \leq 2.306$

$$t = \frac{\bar{X} - \mu}{s_{\bar{X}}} = \frac{(8.54 \times 10^{-2} \text{min}^{-1}) - (1.84 \times 10^{-4} \text{min}^{-1})}{(1.14 \times 10^{-2} \text{min}^{-1})} = 7.396$$

TABLE VI (Continued)

Since $7.396 \geq 2.306$ reject the null hypothesis and assume there is a significant difference between the observed rates of reduction of the ration and the rate of hydrolytic removal of alkylpurines from DNA. $P < .05$.

TABLE VII. THE CALCULATED RATES FOR $^3\text{H}/^{32}\text{P}$ REDUCTION IN THE EMBRYOS
DURING LATER STAGES AND T-TEST OF THE RATES.

Experiment	Time (minutes)	$\frac{^3\text{H}/^{32}\text{P Embryos}}{^3\text{H}^{32}\text{P Sperm}} = \text{N}/\text{N}_0$	$\text{Log}_e \text{N}/\text{N}_0$	$k = \frac{\text{Log}_e \text{N}/\text{N}_0 \text{ (Regression)}}{-\text{time}}^b$
4	0 - 15	0.35	-1.05	
	Mate 1 10 - 25	0.33	-1.11	$3.32 \times 10^{-3} \text{min}^{-1}$
	165 - 180	0.25	-1.61	(S.E. $1.39 \times 10^{-4} \text{min}^{-1}$)
	0 - 15	0.11	-2.21	
Mate 2	10 - 25	0.08	-2.52	$1.06 \times 10^{-3} \text{min}^{-1}$
	165 - 180	0.08	-2.52	(S.E. $1.66 \times 10^{-3} \text{min}^{-1}$)
5	0 - 15	0.31	-1.17	
	Mate 1 10 - 25	0.34	-1.07	$1.65 \times 10^{-3} \text{min}^{-1}$
	165 - 180	0.25	-1.39	(S.E. $6.31 \times 10^{-4} \text{min}^{-1}$)
	0 - 15	0.55	-0.60	
Mate 1	15 - 30	0.55	-0.60	$1.70 \times 10^{-3} \text{min}^{-1}$
Day 1	165 - 180	0.42	-0.87	(S.E. $1.40 \times 10^{-4} \text{min}^{-1}$)

TABLE VII. (Continued)

Experiment	Time (minutes)	$\frac{{}^3\text{H}/{}^{32}\text{P Embryos}}{{}^3\text{H}/{}^{32}\text{P Sperm}} = \text{N}/\text{N}_0$	$\text{Log}_e \text{N}/\text{N}_0$	$k = \frac{\text{Log}_e \text{N}/\text{N}_0 (\text{Regression})^b}{-\text{time}}$
6	Mate 1 0 - 15	0.35	-1.05	0.00
	Day 2 15 - 30	0.35	-1.05	
	165 - 180	0.35	-1.05	
	Mate 1 0 - 15	0.36	-1.02	$-7.21 \times 10^{-5} \text{min}^{-1}$ (S.E. $2.97 \times 10^{-3} \text{min}^{-1}$)
	Day 3 15 - 30	0.21	-1.56	
	165 - 180	0.29	-1.24	

^a Rates calculated only in those matings where 3 observations were made.

^b The k value is computed using intercept model for linear regression ($Y = a + kX$).

Observed rates	Mean	Standard Error	Degrees of Freedom
$3.32 \times 10^{-3} \text{min}^{-1}$			
$1.06 \times 10^{-3} \text{min}^{-1}$			

TABLE VII. (Continued)

Observed rates			Degrees of Freedom
1.65 x 10 ⁻³ min ⁻¹	1.30 x 10 ⁻³ min ⁻¹	5.16 x 10 ⁻⁴ min ⁻¹	5
1.70 x 10 ⁻³ min ⁻¹			
0 min ⁻¹			
-7.21 x 10 ⁻⁵ min ⁻¹			

$$H_0: \mu_0 = 1.84 \times 10^{-4} \text{min}^{-1} = \mu$$

$$H_a: \mu \neq 1.84 \times 10^{-4} \text{min}^{-1}$$

with: $\alpha = 0.05$

limits are: $-2.571 \leq t \leq 2.571$ @ 5df

$$t = \frac{\bar{X} - \mu}{\frac{s_{\bar{X}}}{\sqrt{n}}} = \frac{(1.30 \times 10^{-3} \text{min}^{-1}) - (1.84 \times 10^{-4} \text{min}^{-1})}{(5.16 \times 10^{-4} \text{min}^{-1})} = 2.12$$

Since $-2.571 \leq 2.12 \leq 2.571$, fail to reject the null hypothesis and conclude that there is no significant difference between the observed slow rates of reduction of the ratio and the rate of hydrolytic removal for alkylpurines from DNA ($p < 0.05$).

and 57%, respectively. The semi-log plots of the decrease in ^3H and the calculated Y-intercept values all project a rapid initial decrease (Table VI) in the level of ^3H label. However, the magnitude of this initial decrease differs between all experiments. This difference between experiments is due to differences such as in the method of administering the EMS, in the specific activities of the ^3H -EMS samples, in the type of virgins used for egg collections, etc., but there is no clear pattern to indicate which of the differences influence the magnitude of the initial decrease.

There is a statistically significant difference between the embryonic rates of loss observed prior to the first embryo stages sampled and the rate of spontaneous hydrolytic depurination (Table VI), but there is no significant difference between the observed mean rates of ^3H loss and the rate for hydrolytic depurination of alkylated DNA in the later embryo stages (Table VII). This suggests that between fertilization of the egg and the first or second mitotic division in the embryo, ethyl groups are lost at a rate much greater than the rate for hydrolytic depurination. During this period the alkyl groups are lost at least 400 times faster than would be expected by spontaneous hydrolysis. After the first few mitotic divisions in the early embryo ^3H loss approximates the rate for hydrolytic loss of alkylpurines.

Fertility of Embryos Collected with Carbon Dioxide Gas

When this series of experiments were initially designed it was expected that eggs would be collected by simply allowing the inseminated females to lay on a media specially prepared to induce egg laying (Würgler et al., 1968). This method of egg collection worked well for

the first two experiments in which the earliest embryo samples were three to four hours old (gastrula stage). In these first experiments the females could be left to lay eggs undisturbed for at least one hour. Thus, large groups of eggs having approximately the same embryonic development could be collected easily. In the remaining four experiments it was necessary to collect egg samples over shorter time intervals and to reduce the age range of embryos in each sample. The normal egg laying rate of the inseminated females was not sufficient to allow collection of large numbers of closely timed embryos. It was proposed that these more closely times samples could be collected by using anesthetizing doses of carbon dioxide gas to induce egg laying by the inseminated females.

Since the experiments are designed to investigate embryonic events it is important that the collected eggs be fertilized. Thus, it was necessary to determine how the CO₂ treatment affected the fertility of the sampled eggs. This affect of CO₂ on egg fertility was measured in six independent determinations, in which separate groups of 200-300 inseminated 4-6 day old female flies were used for each determination. The inseminated females were allowed to deposit eggs over one-hour periods on the special media of Würigler et al., (1968). Samples taken prior to CO₂ subjection served as controls. After a sufficient number of control eggs were collected the females were administered anesthetizing doses of CO₂ to induce egg deposition. The females were allowed to recover from the affects of CO₂ for at least one hour before being anesthetized again. Twenty-four hours after deposition the eggs from both the control and CO₂ groups were scored for hatchability (Table VIII).

TABLE VIII. THE EGG FERTILITY USING CARBON DIOXIDE GAS TO INDUCE EGG LAYING

Determination	Control			CO2 Group		
	No. Eggs	No. Hatch	% Hatch	No. Eggs	No. Hatch	% Hatch
1	420	359	85.5	571	383	67.1
2	110	101	91.8	158	120	75.9
3	174	153	87.9	583	375	64.3
4	152	123	80.9	707	408	57.7
5	255	230	90.2	583	362	62.1
6	528	497	94.1	509	408	80.2
$\bar{X}$			88.4			68.0
SD			4.7			8.4

The mean hatchability for the control eggs combining all of the determinations was 88% ($\pm 5\%$ SD). The mean hatchability of the embryos in the CO₂ group was 68% ($\pm 8\%$ SD). Since the majority of the eggs collected with CO₂ were fertile it was decided that further studies could be carried out using CO₂ to collect timed egg samples. Eggs that are not fertilized do not contribute either one of the radionuclides to the DNA extracts and therefore do not affect the ³H/³²P ratios.

Percent of Retained Eggs in Pooled Embryo Samples

Normally, the egg passes into the uterus where it is fertilized by a spermatozoa and the egg is deposited on the media soon after. These two events occur in a very short period of time such that embryonic development is considered to begin when the egg is deposited. Sometimes a female may retain an inseminated egg in the uterus for a period of time. Such an egg begins embryonic development within the female and is deposited in an advanced developmental stage (Würgler et al., 1968). During these experiments an attempt was made to separate the early embryo samples into distinct developmental stages (premitotic, 15 minutes after deposition; first cleavage division, 20 and 25 minutes after deposition; and second division, 30 minutes after deposition) based on the time schedule of Rabinowitz (1941). Thus it is important to determine what proportion of the embryos are retained from one stage to the next.

As described in the Methods and Materials embryos from each stage were selected at random and prepared for cytological examination. The description of early cleavage for Drosophila melanogaster given by Sonnenblick (1965) was used as a cytological guide for determining the

developmental stage of a particular embryo. Approximately 1% of the total egg sample from any particular developmental stage was prepared for cytological examination and these embryos were selected at random from approximately 16% of the egg samples pooled for a particular developmental stage. Table IX gives the results of cytological examinations of embryos from seven different groups of pooled egg samples distributed among four different experiments. Assuming that the proportion of retained eggs in a pooled sample is not dependent on what stage the eggs have reached when development is stopped or in what experiment the samples are collected, a mean value based on all seven observations can be computed. Among the seven groups of pooled embryo samples the overall mean of retained eggs in the cytological samples is 8% ($\pm 6\%$ SD). Since on the average only 68% of the embryos collected are fertile it can be expected, based on cytological examination that as many as 21% of the fertile embryos in a pooled sample may have come from fertilized eggs that had been retained in the uterus of the female. The observation that there might be overlap between embryo stages must be considered when comparing the $^3\text{H}/^{32}\text{P}$ ratios obtained from the various embryonic stages.

The Effect of DNA Source on the Extraction Procedure

The DNA in the sperm cells is bounded by arginine-rich histones, tightly coiled, and in a non-metabolizing condition, while the sperm derived DNA of the embryo is bounded by lysine-rich histones, is not as tightly coiled as the DNA in the sperm cells, and is capable of metabolic activity (Das, et al., 1964a and 1964b; Oliveri and Oliveri, 1965; Brink, 1968). Since the DNA of the sperm is packaged differently

TABLE IX. THE PERCENTAGE OF EMBRYOS RETAINED BEYOND THE STAGE SAMPLED

Experiment	Average age of eggs in the pooled sample (min)	No. eggs in the pooled sample	No. eggs cytologically examined	No. re-tained eggs	Percent ^a retained eggs
4	15	3675	37	3	8.1
	25	3626	44	2	4.5
5	15	6055	40	1	2.5
	25	6397	24	0	0.0
6	15	7600	64	8	13.0
	30	8992	52	6	12.0
7	30	4175	37	6	16.2

^a $\bar{X}$ for the percent of embryos retained equals 8 ± 6 standard deviation.

^b Experiment 7 sample is from the ³H-MMS treatment used to determine the efficiency of the extraction procedure for the two types of DNA sources.

than the sperm derived DNA of the embryo it is possible that the DNA extraction procedure has a differential effect on DNA from the two different sources. It might be suggested that a differential effect of the DNA extraction procedure is responsible for the apparent reduction of ^3H -labeled bases from the DNA of the embryos.

^{32}P -labelled CD males were fed ^3H -methyl methanesulfonate (specific activity 6900 mCi/mM) and mated to Q virgins for one day. After mating the males were separated from the females and frozen. Egg samples were collected from the inseminated females with the use of CO_2 and allowed to develop 30 minutes before freezing on dry ice. The individual embryo samples were pooled to form one large sample of 30 minute eggs. Sperm cells were sampled by removing the seminal vesicles from frozen ^3H -MMS treated males, and pooled to form one vesicle sample. Each of the two samples were placed in a glass homogenizer and ground in a mercapto-ethanol solution (6 ml, 6.7% in 1 x SSC; 0.1 M Tris, pH 8.5) at 4°C until homogenized. If there is a differential removal of ^3H -label by the extraction procedure it would most likely occur when the disrupted cells are in the mercaptoethanol prior to the first separation of the nucleic acids from the associated proteins and cellular homogenate. Beyond this point in the extraction procedure the separated nucleic acids from both sources would have similar characteristics and thus their bases would not be subject to differential removal.

The loss of ^3H -labeled bases from either DNA source in the mercaptoethanol solution can be detected by comparing the $^3\text{H}/^{32}\text{P}$ ratio of final DNA extracts that are allowed to remain in the mercaptoethanol solution for differing lengths of time. After homogenization in the

mercaptoethanol solution DNA extraction procedures were carried out on aliquots of solution incubated for 0, 1, and 4 hours. The ^3H and ^{32}P dpm and the $^3\text{H}/^{32}\text{P}$ ratios for these extracts are given in Table XII. Loss of labeled bases from either the egg or the sperm DNA while in the mercaptoethanol solution would result in a time dependent reduction of ^3H -label in the final DNA extracts. Since the phosphorus moiety of the DNA should not be affected by loss of the labeled bases the $^3\text{H}/^{32}\text{P}$ ratio should decrease if there is a time dependent loss of alkylated bases in the mercaptoethanol solution. The samples from the sperm cell DNA of the vesicles and the sperm derived DNA of the embryos increase in ^3H and ^{32}P dmp with increased time in the mercaptoethanol, but maintained the same $^3\text{H}/^{32}\text{P}$ ratio. This indicates that the efficiency of the DNA extraction was increased with longer exposures to mercaptoethanol, but there was no decrease in the level of ^3H -label in any of the DNA extractions. An ANOV of the $^3\text{H}/^{32}\text{P}$ ratios of DNA derived from the 0, 1, and 4 hour incubations of the sperm and embryos (Table X) indicate that there is no change with increased incubation time in mercaptoethanol. Therefore, the decrease in ^3H -label observed in the embryo samples of the experiments must be due to something other than a differential effect of the extraction procedure.

TABLE X. VERIFICATION OF DNA EXTRACTION PROCEDURE AND F-TEST OF RATIOS

DNA Source	Time in Mercapto- ethanol Solution (hours)	^3H -DPM	^{32}P -DPM	$^3\text{H}/^{32}\text{P}_a$
Embryos	0	237	31	$7.61 \pm .02$
	1	315	44	$7.16 \pm .02$
	4	575	80	$7.14 \pm .01$
Sperm Cells	0	924	407	$2.27 \pm .01$
	1	1515	616	$2.46 \pm .01$
	4	2127	866	$2.45 \pm .01$

^aRelative Standard Deviation based on counting time (sampling error).

TABLE X (Continued)

	Time in Mercaptoethanol		
	0 hr.	1 hr.	4 hr.
Embryos	7.61	7.16	7.14
Sperm cells	2.27	2.46	2.45

ANOVA TABLE

Source	Degrees of Freedom	Sum of Squares	Mean Square	F-test	Probability
Total	5	36.32			
Time	1	0.03	0.030	0.46	NS
Stage	2	36.16	18.080	278.15	P<.01
Error	2	0.13	0.065		

DISCUSSION

The ^3H -ethyl groups attached to the ^{32}P -labeled DNA of the ^3H -EMS treated sperm cells might be affected in several ways following exposure. First, these ethyl groups might remain attached to the DNA derived from sperm during the entire period studied, a period which began with ^3H -EMS treatment of the male, continued through insemination, sperm storage in the female's seminal receptacle, fertilization of the egg, and terminated at various stages of embryonic development. The ^{32}P label is randomly incorporated in the DNA of the sperm cells, therefore loss of the ^{32}P and the chain breaks associated with the ^{32}P decay occur at random with respect to the ^3H -ethyl sites. When the majority of the ethyl groups remain attached to the DNA throughout embryonic development the $^3\text{H}/^{32}\text{P}$ ratio after correction for ^{32}P decay should be constant for all stages sampled (sperm and embryos). No significant difference would be expected between this ratio observed in the DNA of the sperm cells and the ratio in the sperm derived DNA of the embryos.

Another possible occurrence would be the dissociation of the induced ^3H -ethyl groups from the ^{32}P -labeled sperm derived DNA sometime after treatment. This dissociation of ethyl groups might occur in sperm cells while they are stored in either the males or females and/or it could take place in the embryo during development. The loss of ethyl groups from the alkylated sperm derived DNA might result from several causes. Alkyl purines are hydrolytically removed from DNA at a mean rate approximately 1×10^5 times faster than the rate observed

for hydrolytic loss of non-alkylated purines from DNA (Lindahl and Nyberg, 1972; Strauss et al., 1975). Lett et al., (1962) have shown that hydrolysis of the quaternized alkyl purine products in double stranded DNA occurs at the N-glycosidic bond which links the sugar from the DNA backbone to the alkylated purine, but does not frequently occur at the bond which links the alkyl group to the purine. Thus, alkyl purines are hydrolytically split from the DNA but the alkyl groups are not readily hydrolyzed from the alkyl purine. Since the majority of ethylations on DNA occur at the N-7 position of guanine (Lawley and Brookes, 1963; Aaron et al., 1973; Sun and Singer, 1975) a decrease in the level of ^3H -ethyl groups on the DNA would result from the hydrolytic loss of the ethylated purines. The hydrolytic loss of ethylated bases from the alkylated sperm DNA during storage, either in the seminal vesicles of the males or the seminal receptacles of the females, should cause the $^3\text{H}/^{32}\text{P}$ ratio of stored sperm DNA to undergo a time dependent reduction. The magnitude of this reduction would be directly related to the amount of ^3H -ethylated bases lost, and since the majority of alkylations in the *Drosophila* DNA occur on the purine sites (Aaron et al., 1973) the rate of reduction of this ratio should not be significantly different from the estimated depurination rate for alkylated DNA. The hydrolytic loss of ethylated bases during embryonic development could also result in a time dependent reduction in the embryonic $^3\text{H}/^{32}\text{P}$ ratio by approximately the rate of DNA depurination for alkylated bases.

The loss of ethyl groups might also result from an active enzymatic removal of alkylated bases from the DNA (Strauss and Robins, 1976;

Lawley and Orr, 1970; Margison and O'Connor, 1973). An enzymatic removal of ^3H -ethyl groups or the ^3H -ethylated bases from treated sperm derived DNA via some repair system must occur at a rate greater than the spontaneous hydrolysis rate for depurination of alkylated bases if it is to be detected as a different process. The onset of such an enzymatic loss of the ^3H -labeled ethyl groups should result in a decrease in the $^3\text{H}/^{32}\text{P}$ ratio of the sperm derived DNA, either during storage or embryonic development, at rates significantly faster than the rate for depurination of alkylated DNA.

It is apparent from examination of the $^3\text{H}/^{32}\text{P}$ ratios of sperm cells stored in seminal receptacles of female flies for short and long periods of time that the ratio does not remain constant during storage (Table IV and Figure 4). During these periods of storage (0-7 days) the ratio decreased at approximately the rate expected for the hydrolytic loss of a alkylated purines from DNA. The observed mean rate in the reduction of % ^3H from the DNA of stored sperm (Figure 4) is $1.61 \times 10^{-4} \text{min}^{-1}$. This value does not appear to differ from the hydrolytic depurination rate of $1.84 \times 10^{-4} \text{min}^{-1}$ (Strauss et al., 1975). Because this rate of loss is slow and because insemination of the female and fertilization of the egg occurs one to two days after treatment a large proportion of the alkyl groups accumulated in the treated spermatozoa are retained until fertilization.

If this apparent loss of ^3H in stored sperm cells is due to a hydrolytic depurination of ethylated bases; it is most probable that this hydrolysis begins with the onset of base alkylation at the time of male treatment, and would continue until all the alkylated bases are removed. It would, therefore, be expected that the sperm derived DNA of the

early embryos, exhibit at least a hydrolytic loss of ethylated bases and a corresponding decrease in the $^3\text{H}/^{32}\text{P}$ ratio.

A within experiment comparison of the $^3\text{H}/^{32}\text{P}$ ratios from extracted DNA of sperm cells with the ratio from DNA of embryos shows that the ratio in the embryos is significantly decreased from the ratio in the sperm cells (Table II). This decrease in the isotopic ratio is observed within the earliest embryo stage sampled and continues to be observed throughout the later embryo stages (Table 1, Table III, and Figure 5). Since the DNA extraction procedure extracts DNA non-preferentially from the two sources (Table X) and since the alkyl phosphotriesters formed in the DNA are apparently stable products (Bannon and Verly, 1972) the difference between the sperm and the embryos must represent an active removal of the ^3H which is attached to the ethyl groups on the ethylated DNA bases. The data available does not allow the distinction between 1) loss of the ^3H -ethyl group from ethylated bases of the DNA and 2) removal of the entire ethylated base from the DNA. However, based on the observations of other investigators (Bannon and Verly, 1972; Lett et al., 1962) the latter condition seems more likely.

The release of the ^3H -label in the embryos occurs in two phases (Figure 5). Between fertilization of the egg and the first few embryonic mitotic divisions the loss of ^3H -label from embryo DNA occurs at a mean rate approximately 400 times greater than the rate for spontaneous hydrolytic depurination of alkylated DNA. The data in Table 1 indicates that this very rapid loss of ^3H -label may have occurred prior to the earliest embryo stages sampled (eggs collected 15 and 20 minutes

after deposition) and before the onset of the first mitotic division. The difference between $^3\text{H}/^{32}\text{P}$ ratios observed in sperm and the earliest stage measured in the embryo would require a rate of selective loss of ^3H versus randomized loss of ^{32}P that is at least as high as $8.4 \times 10^{-2} \text{min}^{-1}$ (Table VI). This minimum estimate for the loss of ^3H during the short interval between sperm penetration of the egg and the first embryo sample is significantly faster than could be accounted for by spontaneous hydrolysis of ethylated purines from DNA (Table VI).

The second phase of the ^3H -loss appears to occur after the first or second mitotic division. During the period from the first mitotic division to gastrula formation the ^3H -label is lost at about 0.02 times the rate observed in the first phase. The t-test analysis (Table VII) indicates that the mean rate for this slow phase ($1.30 \times 10^{-3} \text{min}^{-1}$) based on six observations is not significantly different from the rate of hydrolysis for alkylated purines. The slow phase rate was computed using the 15 minute embryo samples as the initial point in the regression. However, due to selective loss of over half of the initial alkylations, the 15 minute embryo samples may have a distribution of alkyl sites quite different from the initial distribution of alkyl sites in the sperm DNA. Thus, this slow phase rate may be biased downward because of a higher proportion of stable alkylation sites.

EMS induction of mutations after treatment of *Drosophila* males is stage specific. The highest mutation frequencies occur when germ cells are treated as late spermatids or mature spermatozoa (Fahmy and Fahmy, 1957 and 1951; Alderson, 1965; Epler, 1966; Jenkins, 1967a and 1967b). Treatment of these stages results in the accumulation of high levels

of alkylation on the sperm DNA but produce comparatively low frequencies of sex-linked recessive mutations in the progeny of treated males (Aaron and Lee, 1977). A high frequency of the EMS induced mutations are mosaic mutations (Jenkins, 1967a and 1967b; Epler, 1966; Alderson, 1967; Lee et al., 1970). Analysis of the distribution of mosaics from several experimental sources has led Lee (1976) to estimate that on the average mosaic embryos carry a mutation in only 1/4 of their nuclei. Finally, there is an apparent direct relationship between the time treated sperm cells are stored in the seminal receptacles and the frequency of translocations, chromosome loss and dominant lethals observed in the progeny (Abrahamson et al., 1969; Srám, 1970a and 1970b; Schalet, 1977).

There is evidence from several sources for the existence of cellular enzymes which can recognize and remove alkylated DNA bases (Strauss and Robins, 1967; Kirtikar and Goldthwait, 1974; Lawley and Orr, 1970; Margison and O'Connor, 1973). In addition it has been shown that alkylated bases are hydrolyzed off alkylated DNA at rates significantly greater than those for hydrolytic loss of the corresponding non-alkylated bases (Lindahl and Nyberg, 1972; Lawley and Brookes, 1963; Margison and O'Connor, 1973; Strauss et al., 1975). Enzymatic removal and hydrolytic removal both leave apurinic and possibly apyrimidinic sites in DNA at the point of base removal. These apurinic and apyrimidinic DNA sites are unstable and could be further hydrolyzed to form single-strand breaks in the DNA (Strauss and Hill, 1970; Ross, 1958). Endonucleases specific for apurinic (apyrimidinic) sites have been isolated from cellular extracts of bacteria, higher plants and mammals (Verly and Rassart, 1965; Verly et al., 1973; Lindahl and Andersson,

1972; Ljungquist and Lindahl, 1974; Verly and Paquette, 1973). These endonucleases recognize apurinic sites, hydrolyze phosphodiester bonds near each apurinic site and thus introduce single strand breaks into the DNA. Verly et al., (1974) suggests that these endonucleases are part of an enzymatic system which repairs the naturally occurring in vivo depurination of cellular DNA. The exact nature of this enzymatic system is not known, but there is in vitro evidence to suggest that excision repair processes do repair alkylation damage in DNA (Verly et al., 1974). Boyd and Presley (1974) have observed that EMS induces in vivo unscheduled DNA replication in mitotic cells of *Drosophila* larvae. Unscheduled DNA synthesis would be expected to result when a cellular excision-repair system operates to repair damaged DNA. Boyd and Setlow (1975) and Boyd et al. (1975) also provide evidence that certain MMS sensitive strains of *Drosophila melanogaster* are deficient for normal excision-and post-replication repair processes. Both of these processes would be enzyme mediated.

The following sequence of events is proposed to account for the various aspects of the EMS mutation spectrum in *Drosophila* and the apparent shift in the $^3\text{H}/^{32}\text{P}$ ratio observed between sperm DNA and sperm derived DNA of embryos. The sequence is based on the assumption that among the "normal complement" of *Drosophila* cellular enzymes there exists a system that has the capability of recognizing, removing and repairing DNA damage resulting from alkylation of the nitrogenous DNA bases. 1) During spermatogenesis the early cell stages (spermatogonia to early spermatids) are capable of enzymatically repairing DNA alkylation damage induced by EMS treatment of the males. As a result the level of promutagenic and/or mutagenic effects which could

accumulate are kept to a minimum. 2) Upon undergoing the metabolic, physiologic and morphologic changes which transform spermatids to spermatozoa; spermatids lose the ability to rapidly enzymatically repair DNA alkylation damage. Late spermatids and mature spermatozoa are, therefore, incapable of carrying out this repair. It had earlier been shown that the ability to rejoin chromosomes broken by ionizing radiation was lost in late spermatids and spermatozoa. As a result of losing this repair ability alkylations accumulate to relatively high levels on the DNA in these cell stages. During these stages hydrolytic loss of alkylated bases could occur. Since the majority of the alkylations are expected to occur on purine bases (Aaron et al., 1973; Lawley and Brookes, 1963; Sun and Singer, 1975) the rate of loss of alkylated bases would be expected to approximate the rate predicted for hydrolytic loss of alkyl purines ($1.84 \times 10^{-4} \text{min}^{-1}$), and would account for the decrease in the $^3\text{H}/^{32}\text{P}$ ratios observed during sperm storage. Hydrolytic depurination of the alkyl purines from the sperm DNA would lead to a time dependent accumulation of apurinic sites. These unstable sites might be further hydrolyzed to single-strand DNA breaks in the sperm or be retained as apurinic sites until fertilization. 3) The ovum contains finite levels of enzymes required for repairing alkylation DNA damage, and therefore after fertilization the accumulated alkylated bases and alkylation induced damage (apurinic sites and single-strand breaks) introduced on the sperm derived DNA are subject to rapid repair. It is also possible that the mature spermatozoa contains repair enzymes, which are unable to function in the sperm but can function in the environment of the newly fertilized egg because the substrate (alkylated DNA) becomes receptive to the

enzymes.

The Effect of Enzyme Repair on Recessive Mutation Induction

The mutation response observed in *Drosophila* for the EMS induction of sex-linked recessive lethals can be accounted for if there are two different repair mechanisms available to repair alkylation damage. The first system is an error-free repair system which removes induced alkylations in the DNA of the spermatogonia and the early stages of spermatogenesis (spermatocytes to early spermatids). This repair system would reduce the initial level of alkylation and the mutagenic potential of alkylation at these stages such that the induced frequency of mutations is not significantly increased from the control rate. The second system is an error-prone repair system which removes alkylation damage in the sperm pronucleus prior to the first cleavage division in the embryo. This mechanism would have a low finite probability (b) of producing a mutagenic alteration each time it repairs a site of alkylation damage. The frequency of induced mutations (e.g. sex-linked recessive lethal mutations) would therefore be linearly related to the level of alkylation such that;

$m = bX$ = the mutation frequency induced,

where; b = probability of repair inducing a mutation

and; X = level of alkylation (alkylation per nucleotide).

These two repair systems, an error-free system operating primarily in the early stages of spermatogenesis and an error-prone system operating prior to the first cleavage division in the embryo, could account for the germ stage specificity for inducing recessive mutations (Fahmy and Fahmy, 1957 and 1961; Alderson, 1965; Epler, 1966; Jenkins, 1967a and

1967b), the germ stage specificity for induced DNA alkylation (Aaron et al., 1973), and the apparent inefficiency with which DNA alkylation in spermatozoa are converted to mutations (Aaron and Lee, 1978). Most importantly, the relationship between alkylation and genetic response predicted by the sequential operation of the two repair systems is consistent with the linear dose-response relationship observed by Aaron and Lee, (1978).

Effect of Accumulation of Apurinic sites on Mutation Induction

A time dependent accumulation of alkylation damage on sperm DNA could result in the increasing frequency of chromosome aberrations observed with storage of treated sperm cells (Abrahamson et al., 1969; Srám, 1970a and 1970b; Schalet, 1977). After EMS treatment the accumulation of apurinic sites in the DNA as a result of hydrolysis of alkylated bases is time dependent. Therefore, stored sperm DNA should contain more apurinic sites than DNA from non-stored sperm. Increasing the level of apurinic sites should also increase the probability of introducing hydrolytically induced single-strand breaks in the treated DNA of stored sperm (Strauss and Hill, 1970). Also, the efficiency of the repair for apurinic sites in the embryos would depend on the concentration of the repair enzymes and the concentration of the apurinic sites in the alkylated DNA. Therefore, it is possible that as a result of storage, apurinic sites in the alkylated DNA could accumulate to such high levels that during the early embryo period the repair system could not complete repair on all the apurinic sites present in the alkylated sperm derived DNA. The activity of an endonuclease specific for apurinic sites without subsequent or concurrent repair activity

could introduce single-strand breaks into the alkylated sperm derived DNA. Alternately, apurinic sites which are retained throughout the repair period in the embryo could be hydrolyzed at a later time to introduce single-strand breaks into the alkylated DNA. During the first round of DNA synthesis in the embryo any remaining single-strand breaks could be converted to chromatid breaks. Subsequently, these chromatid breaks can be converted to the types of chromosome aberrations observed in progeny of stored treated sperm cells. Thus, increasing the time of sperm storage could result in an increased potential for chromosome aberrations in the genome of the progeny.

The Effect of Alkylation on the Nuclear Complement of the Embryo

Fertilization in *Drosophila* is a gonameric process in which both the haploid sperm cell and the haploid fertilization nucleus of the egg undergo a mitotic division prior to fusion (Heuttner, 1924; Sonnenblick, 1965). The egg and sperm pronuclei are aligned side by side on the metaphase plate but remain separate until immediately following anaphase of this first mitotic division. At this time the products of mitosis fuse yielding two diploid embryonic nuclei each containing a maternal and paternal genetic complement. The first DNA synthesis in the embryo probably occurs prior to the fusion of the daughter pro-nuclei. Individual EMS alkylations occur on only one of the two strands of a DNA molecule. If the original single-strand DNA alkylation damage is converted to a double-strand chromosome alteration prior to the first DNA synthesis both daughter sperm pronuclei will carry the alteration. Depending on the nature of the alteration the resulting embryo may carry a mutation in all of its nuclei or be mosaic for the mutation. When

the chromosome alteration is a mutagenic alteration, the resulting embryo would carry the mutation in all its nuclei. Alternately, if the double-strand chromosome alteration is a promutagenic alteration (an alteration which has the potential of producing a mutagenic alteration at a later time) the embryo, if mutant, would be mosaic for the mutation. Since the majority of the EMS induced mutations are mosaic, conversion of single-strand alkylation damage to a double-strand mutagenic alteration prior to the first DNA synthesis probably occurs infrequently. Unrepaired or mis-repaired single-strand alkylation damage on the sperm derived DNA retained through the first DNA synthesis would induce a mutagenic or promutagenic event in only one of the two daughter sperm pronuclei. The other sperm pronucleus would contain no alteration at the corresponding DNA site. Thus, after fusion of the Egg and sperm pronuclei only one of the two diploid embryo nuclei would carry a mutagenic or promutagenic alteration at an original alkylated site. Retention of single-strand alkylation damage through the first DNA synthesis or mis-repair of alkylation damage, can produce mosaic embryos. Single-strand chromatid breaks retained after the first DNA synthesis can result in embryos which are mosaic for chromosome aberrations, but because of the small number of nuclei which give rise to the germ line (Sonnenblick, 1965) and because of the poor mixing of embryonic nuclei from which the germ line nuclei are drawn resulting in the effective number of mutant nuclei being 1.3 (Lee, et al., 1970; Lee, 1976), most mosaic individuals will be scored as complete (individuals that contain a completely mutant or non-mutant germ line) when judged by mutation transmission. Retention of single-strand alkylation damage and/or mis-repair can also produce a complete mutant embryo. This can occur if

one of the two embryonic nuclei formed after pronuclei fusion contains a single-strand break or another mutagenic alteration, while the other nucleus contains a dicentric bridge or any other configuration which causes early lethality (Leigh, 1976). If the mutagenic alteration in the one embryonic nucleus is viable the embryo will develop and all its nuclei will carry the mutagenic alteration from the original viable embryonic nucleus.

The frequency of mutant carrying nuclei in a mosaic embryo is dependent on the time during embryonic development at which alkylation damage is converted to a mutation. When the mutation is fixed after the first DNA synthesis 50% of the nuclei should carry the mutation, while mutations that are fixed after the second round of DNA synthesis would be carried by only 25% of the embryonic nuclei, etc. On the average only 1/4 of the nuclei of mosaic embryos carry recessive mutations (Lee, 1976). This indicates that the majority of recessive mutations may not be fixed until the second or later rounds of DNA synthesis in the embryo.

CONCLUSIONS

1. Storage of ^3H -EMS treated Drosophila melanogaster sperm cells in the seminal receptacles of inseminated females results in a time dependent decrease in the level of induced ^3H -ethyl groups on the sperm DNA.
2. The rate of decrease in the ^3H -labeled alkyl groups is slow and does not appear to be greater than the rate predicted for hydrolytic loss of alkyl purines ($1.84 \times 10^{-4} \text{min}^{-1}$) from DNA.
3. Between the time of fertilization and the first or second cleavage divisions in the embryo (21-33 minutes after fertilization) the level of alkyl groups on the sperm derived DNA is reduced by a rate approximately 400 times greater than that expected for hydrolytic loss of alkyl purines.
4. Beyond this stage of embryonic development the level of alkyl groups continues to decrease, but the rate of loss is not significantly different from that expected for the hydrolytic depurination of alkylated DNA.
5. Even though alkyl groups are lost during the earlier and later stages of the embryonic syncytium, detectable levels of ^3H -labeled ethyl groups are still observed when the embryo undergoes cellularity at the gastrula stage (180 minutes after fertilization).

LITERATURE CITATION

- Aaron, C.S., and Lee, W. R., (1975) Computerized liquid scintillation counting data management, 1. Time sequence for decay correction: A key for data storage and retrieval, Analytical Biochem. 65: 573-577.
- Aaron, C.S., and Lee, W.R., (1977) Molecular dosimetry of the mutagen ethyl methanesulfonate in Drosophila melanogaster spermatozoa: Linear relation of DNA alkylation per sperm cell (dose) to sex-linked recessive lethals, (Abstract) Proceedings of the 8th Annual Meeting of the Environmental Mutagen Society in Colorado Springs, Co.
- Aaron, C.S., and Lee, W.R., (1978) Molecular dosimetry of the mutagen ethyl methanesulfonate in Drosophila melanogaster spermatozoa: Linear relation of DNA alkylation per sperm cell (dose) to sex-linked recessive lethals, Mutation Res. 49:27-44.
- Aaron, C.S., Lee, W.R., Janca, F.C., and Seamster, P.M., (1973) Ethylation of DNA in the germ line of male Drosophila melanogaster, (Abstract) Proceedings of the 13th International Congress of Genetics, Berkeley, California, Genetics 74:1s.
- Aaron, C.S., Nardin, H.E., and Lee, W.R., (1977a) Glass filter supports for treatment of adult D. melanogaster with chemical mutagens, Drosophila Information Service 52:166.
- Aaron, C.S., Lee, W.R., Seamster, P.M., and Janca, F.C., (1977) A non-aqueous gas exposure technique for microliter quantities of chemical mutagens, Drosophila Information Service 52:174.
- Abrahamson, S., Kiriazis, W.C., and Sabol, E.M., (1969) A storage effect of ethyl methanesulfonate (EMS) on the induction of translocations in Drosophila sperm, Drosophila Information Service 44:110-111.
- Alderson, T., (1965) Chemically induced delayed germinal mutation in Drosophila, Nature 207:164-166.
- Amano, E., and Smith, H.H., (1965) Mutations induced by ethyl methane-sulfonate in Maize, Mutation Res. 2:344-351.
- Auerbach, C., (1946) Chemically induced mosaicism in Drosophila melanogaster, Proc. R. Soc. Edinb. B62:211-221.
- Ayad, S.R., Fox, M., and Fox, B.W., (1969) Non-semiconservative incorporation of labeled 5-bromo-2'-deoxyuridine in lymphoma cells treated with low doses of methyl methanesulfonate, Mutation Res. 8:639-645.

- Bannon, P., and Verly, W., (1972) Alkylation of phosphates and stability of phosphate triesters in DNA, Eur. J. Biochem. 31:103-111.
- Bender, M.A., Griggs, H.G., and Bedford, J.S., (1974) Mechanisms of chromosomal aberration production, III. Chemicals and ionizing radiation, Mutation Res. 23:197-212.
- Bhatia, C.R., (1967) Increased mutagenic effect of ethyl methanesulfonate when dissolved in dimethyl sulfoxide, Mutation Res. 4:375-376.
- Boyd, J.B., Golino, M.D., and Setlow, R.B., (1976b) The mei-9^a mutant of Drosophila melanogaster increases mutagen sensitivity and decreases excision repair, Genetics 84:527-544.
- Boyd, J.B., and Presley, J.M., (1974) Repair replication and photo-repair of DNA in larvae of Drosophila melanogaster, Genetics 77: 687-700.
- Boyd, J.B., and Setlow, R.B., (1976a) Characterization of postreplication repair in mutagen-sensitive strains of Drosophila melanogaster, Genetics 84:507-526.
- Brink, N.G., (1968) Protein synthesis during spermatogenesis in Drosophila melanogaster, Mutation Res. 5:192-194.
- Brookes, P., and Lawley, P.D., (1963) Effects of alkylating agents on T2 and T4 bacteriophages, Biochem.J. 89:138-144.
- Buhl, S.N., and Regan, J.D., (1973) DNA replication in human cells treated with methyl methanesulfonate, Mutation Res. 18:191-197.
- Cattanach, B.M., Pollard, C.E., and Isaacson, J.H., (1968) Ethyl methanesulfonate induced chromosome breakage in the mouse, Mutation Res. 6:297-307.
- Chu, E.H.Y., and Malling, H.V., (1968) Mammalian cell genetics, II. Chemical induction of specific locus mutations in Chinese hamster cells in vitro, Proc. Nat. Acad. Sci. U.S. 61:1306-1312.
- Cooper, K.W., (1965) Normal spermatogenesis in Drosophila, in: Biology of Drosophila, (M. Demerec, ed.) Hafner Publishing Co., New York, 1-61.
- Das, C.C., Kaufmann, B.P., and Gay, H., (1964a) Histone-protein transition in Drosophila melanogaster, I. Changes during spermatogenesis, Exp. Cell Res. 35:507-514.
- Das, C.C., Kaufmann, B.P., and Gay, H., (1964b) Autoradiographic evidence of synthesis of an arginine-rich histone during spermiogenesis in Drosophila melanogaster, Nature 204:1008-1009.

- Drake, J.W., (1970) The Molecular Basis of Mutation, Holden-Day, San Francisco.
- Ehling, U.H., Cummings, R.B., and Malling, H.V., Induction of dominant lethal mutations by alkylating agents in male mice, Mutation Res. 5:417-428.
- Epler, J.L., (1966) Ethyl methanesulfonate-induced lethals in *Drosophila*, Frequency-dose relations and multiple mosaicism, Genetics 54:31-36.
- Fahmy, O.G., and Fahmy, M.J., (1957) Mutagenic responses to alkyl methanesulfonate during spermatogenesis in *Drosophila melanogaster*, Nature 180:31-34.
- Fahmy, O.G., and Fahmy, M.J., (1961) Cytogenetic analysis of the action of carcinogens and tumor inhibitors in *Drosophila melanogaster*, IX. The cell stage response of the male germ line to the mesyloxy esters, Genetics 46:361-372.
- Ficsor, G., (1965) The nature and expression of ethyl methanesulfonate (EMS), diethyl sulfate (DES), X-rays, and UV-induced mutations in *Zea mays*, Proceedings of the Symposium on the Mutational Process, Prague August 9-11:163-168.
- Fishbein, L., Flamm, W.G., and Falk, H.L., (1970) Chemical Mutagens, Environmental Effects on Biological Systems, Academic Press, New York.
- Freese, E., (1963) Molecular mechanisms of mutations, in: Molecular Genetics (J.H. Taylor, ed.), Part 1, Academic Press, New York, 207-269.
- Generoso, W.M., and Russell, W.L., (1969) Strain and sex variations in the sensitivity of mice to dominant-lethal induction with ethyl methanesulfonate, Mutation Res. 8:589-598.
- Generoso, W.M., Russell, W.L., Huff, S.W., Stout, S.K., and Gosslee, D.G., (1974) Effects of Dose on the Induction of Dominant-Lethal Mutations and Heritable Translocations with Ethyl Methanesulfonate in Male Mice, Genetics 77:741-752.
- Gerchman, L.L., and Ludlum, D.B., (1973) The properties of ⁰⁶-methyl-guanine in templates for RNA polymerase, Biochim. Biophys. Acta. 308:310-316.
- Grossman, L., (1975) Excision repair of DNA, in: DNA synthesis and Its Regulation, (M. Goulian and P.C. Hanawalt, eds.; C.F. Fox, series ed.), W.A. Benjamin Inc., Menlo Park, 791-814.
- Higgins, N.P., Kato, K., and Strauss, B., (1976) A model for replication repair in mammalian cells, J. Mol. Biol. 101:417-425.

- Hildreth, P.E., and Lucchesi, J.C., (1963) Fertilization in *Drosophila*: 1. Evidence for the regular occurrence of monospermy, Developmental Biology 6:262:278.
- Huettner, A.G., (1924) Maturation and fertilization in *Drosophila melanogaster*, J. Morph. Physiol. 39:249-265.
- Jenkins, J.B., (1967a) The induction of mosaic and complete dumpy mutants in *Drosophila melanogaster* with ethyl methanesulfonate, Mutation Res. 4:90-92.
- Jenkins, J.B., (1967b) Mutagenesis at a complex locus in *Drosophila* with the monofunctional agent ethyl methanesulfonate, Genetics 57:783-793.
- Krieg, D.R., (1963) Ethyl methanesulfonate-induced reversion of bacteriophage T4rII mutants, Genetics 48:561-580.
- Kirtikar, D.M., and Goldthwait, D.A., (1974) The enzymatic release of O-6-methylguanine and 3-methyladenine for DNA reacted with the carcinogen N-methyl-N-nitrosourea, Proc. Nat. Acad. Sci. U.S. 71:2022-2026.
- Laird, C.D., (1973) DNA of *Drosophila* chromosomes, Annual Review of Genetics 7:177-204.
- Lawley, P.D., (1966) Effects of some chemical mutagens and carcinogens on nucleic acids, in: Progress in Nucleic Acid Research and Molecular Biology (J.N. Davidson and W.E. Cohn, eds.), Academic Press, New York, 5:89-131.
- Lawley, P.D., (1974a) Some chemical aspects of dose-response relationships in alkylation mutagenesis, Mutation Res. 23:283-295.
- Lawley, P.D., (1974b) Alkylation of nucleic acids and mutagenesis, in: Molecular and Environmental Aspects of Mutagenesis (L. Prakash, F. Sherman, M.W. Miller, E.W. Lawrence, and H.W. Taber, eds.), Proceedings of the Sixth Rochester International Conference on Environmental Toxicity, Charles C. Thomas, Publisher, Springfield, 17-33.
- Lawley, P.D., (1975) "Excision" of bases from DNA methylated by carcinogens in vivo and its possible significance in mutagenesis and carcinogenesis, in: Molecular Mechanisms for Repair of DNA (P.C. Hanawalt and R.B. Setlow, eds.; A. Holleander, series ed.), Part A, Plenum Press, New York, 25-28.
- Lawley, P.D., and Brookes, P., (1961) Acidic dissociation of 7:9-dialkylguanines and its possible relation to mutagenic properties of alkylating agents, Nature 192:1080-1082.
- Lawley, P.D., and Brookes, P., (1963) Further studies on the alkylation of nucleic acids and their constituent nucleotides, Biochem. J. 89: 127-138.

- Lawley, P.D., and Orr, D.J., (1970) Specific excision of methylation products from DNA of Escherichia coli treated with N-methyl-N'-nitro-N-nitrosoguanidine, Chem.-Biol. Interactions 2:154-157.
- Lawley, P.D., and Thatcher, C.J., (1970) Methylation of deoxyribonucleic acid in cultured mammalian cells by N-methyl-N'-nitro-N-nitrosoguanidine: The influence of cellular thiol concentrations on the extent of methylation and the 6-oxygen atom of guanine as a site of methylation, Biochem. J. 116:693-707.
- Lee, W.R., (1975) Comparison of the mutagenic effects of chemicals and ionizing radiation using Drosophila melanogaster test systems, in: Radiation Research, Biomedical, Chemical and Physical Perspectives (O.F. Nygaard, H.I. Adler and W.K. Sinclair, eds.), Academic Press, New York, 976-983.
- Lee, W.R. (1976) Chemical mutagenesis, in: The Genetics and Biology of Drosophila (M. Ashburner and E. Novitski, eds.), Academic Press, New York, 1299-1341.
- Lee, W.R., Sega, G.A., and Benson, E.S., (1972) Transmutation of carbon-14 within DNA of Drosophila melanogaster spermatozoa, Mutation Res. 16:195-201.
- Lee, W.R., Sega, G.A., and Bishop, J.B., (1970) Chemically induced mutations observed as mosaics in Drosophila melanogaster, Mutation Res. 9:323-336.
- Leigh, B., (1976) Ring Chromosomes and Radiation Induced Chromosome Loss, in: The Genetics and Biology of Drosophila (M. Ashburner and E. Novitski, eds.) Volume 1b, Academic Press, New York, N.Y., 505-528.
- Lewis, E.B., and Bacher, F., (1968) Method of feeding ethyl methane-sulfonate (EMS) to Drosophila males, Drosophila Information Service 43:193.
- Lindahl, T., and Andersson, A., (1972) Rate of chain breakage at apurinic sites in double-stranded deoxyribonucleic acid, Biochem. 11:3618-3623.
- Lindahl, T., and Nyberg, B., (1972) Rate of depurination of native deoxyribonucleic acid, Biochem. 11:3610-3618.
- Lindsley, D.L., and Grell, E.H., (1972) Genetic Variations of Drosophila melanogaster, Carnegie Institution of Washington Publication No. 627.
- Ljungquist, S., and Lindahl, T., (1974) A mammalian endonuclease specific for apurinic sites in double-stranded deoxyribonucleic acid, I. Purification and general properties, J. Biol. Chem. 249:1530-1535.

- Lett, J.T., Parkins, G.M., and Alexander, P., (1962) Physicochemical changes produced in DNA after alkylation, Archives of Biochemistry and Biophysics 97:80-93.
- Loveless, A., (1966) Genetic and Allied Effects of Alkylating Agents, The Pennsylvania State University Press, University Park.
- Loveless, A., (1969) Possible relevance of O-6 alkylation of deoxyguanosine to mutagenicity and carcinogenicity of nitrosamines and nitrosamides, Nature, 223:206-207.
- Loveless, A., and Howarth, S., (1959) Mutation of bacteria at high levels of survival by ethyl methanesulfonate, Nature 184:1780-1782.
- Ludlum, B., (1970) The properties of 7-methylguanine-containing templates for ribonucleic acid polymerase, J. Biol. Chem. 245:447-482.
- Malling, H.V., and de Serres, F.J., (1968) Identification of genetic alterations induced by ethyl methanesulfonate in Neurospora crassa, Mutation Res. 6:181-193.
- Margison, G.P., and O'Connor, P.J., (1973) Biological implications of the instability of the N-glycosidic bond of 3-methyldeoxyadenosine in DNA, Biochim. Biophys. Acta. 331:349-356.
- Miller, A., (1965) The Internal anatomy and histology of the imago of Drosophila melanogaster, in: Biology of Drosophila (M. Demerec, ed.) Hafner Publishing Co., New York, N.Y., 518-530.
- Olivieri, G., and Olivieri, A., (1965) Autoradiographic study of nucleic acid synthesis during spermatogenesis in Drosophila melanogaster. Mutation Res. 2:366-380.
- Oster, I.I., and Balaban, G., (1963) A modified method for preparing somatic chromosomes, Drosophila Information Service 37:142-143.
- Osterman-Golkar, S., Ehrenberg, L., and Wachtmeister, C.A., (1970) Reaction kinetics and biological action in barley of mono-functional methanesulfonic esters, Radiation Botany 10:303-327.
- Rabinowitz, M., (1941) Studies on the cytology and early embryology of the egg of Drosophila melanogaster, J. Morphol. 69:1-49.
- Rao, R.N., and Natarajan, A.T., (1965) Mutagenicity of some alkyl alkanesulfonates in barley, Mutation Res. 2:132-148.
- Regan, J.D., and Setlow, R.B., (1973) Repair of chemical damage to human DNA, in: Chemical Mutagens; Principles and Methods for Their Detection (A. Hollender, ed.) Plenum Press, New York, 3:151-169.
- Roberts, J.J., Crathorn, A.R., and Brent, T.P., (1968) Repair of alkylated DNA in mammalian cells, Nature 218:970-972.

- Roberts, J.J., Pascoe, J.M., Smith, B.A., and Crathorn, A.R., (1971) Quantitative aspects of the repair of alkylated DNA in cultured mammalian cells, II. Non-semiconservative DNA synthesis ('Repair Synthesis') in HeLa and Chinese hamster cells following treatment with alkylating agents, Chem. Biol. Interactions 3:49-68.
- Ross, W.C.J., (1958) In vitro reactions of biological alkylating agents, Ann. N.Y. Acad. Sci. 68:669-681.
- Schalet, A., (1977) Further evidence of EMS-induced rearrangements and a storage effect in Drosophila melanogaster, (Abstract) Genetics 86:s55.
- Scudiero, D., Henderson, E., Norin, A., and Strauss, B., (1975) The measurement of chemically-induced DNA repair synthesis in human cells by BND-cellulose chromatography, Mutation Res. 29:473-488.
- Sega, G.A., (1974) Unscheduled DNA synthesis in the germ cells of male mice exposed in vivo to the chemical mutagen ethyl methanesulfonate, Proc. Nat. Acad. Sci. U.S. 71:4955-4959.
- Sega, G.A., (1976) Molecular dosimetry of chemical mutagens-measurement of molecular dose and DNA repair in mammalian germ cells, Mutation Res. 38:317-326.
- Shooter, K.V., Edwards, P.A., and Lawley, P.D., (1971) Action of Mono- and difunctional sulphur mustards on the RNA-containing bacteriophage μ 2, Biochem. J. 125:829-840.
- Shooter, K.V., Howse, R., and Merrifield, R.K., (1974b) The reaction of alkylating agents with bacteriophage R17: Biological effects of phosphotriester formation, Biochem. J. 137:313-317.
- Shooter, K.V., Howse, R., Shar, S.A., and Lawley, P.D., (1974a) The molecular basis for biological inactivation of nucleic acids: The action of methylating agents on the ribonucleic acid-containing bacteriophage R17, Biochem. J. 137:303-312.
- Singer, B., (1976) All oxygens in nucleic acids react with carcinogenic ethylating agents, Nature 264:333-339.
- Srám, R.J., (1970a) The effect of storage on the frequency of dominant lethals in Drosophila melanogaster, Molec. Gen. Genetics 106: 286-288.
- Srám, R.J., (1970b) The effect of storage on the frequency of translocations in Drosophila melanogaster, Mutation Res. 9:243-244.
- Sonnenblick, B.P., (1965) The early embryology of Drosophila melanogaster, in: Biology of Drosophila (M. Demerec, ed.) Hafner Publishing Co., New York, 62-167.

- Strauss, B.S., (1961) Specificity of mutagenic action of the alkylating agents, Nature 191:730-731.
- Strauss, B.S., and Hill, T., (1970) The intermediate in the degradation of DNA alkylated with a monofunctional alkylating agent, Biochim. Biophys. Acta. 213:14-25.
- Strauss, B.S., and Robbins, M., (1968) DNA methylated in vitro by a monofunctional alkylating agent as a substrate for a specific nuclease from Micrococcus lysodeikticus, Biochim. Biophys. Acta. 161:68-75.
- Strauss, B., Scudiero, D., and Henderson, E., (1975) The nature of the alkylation lesions in mammalian cells, in: Molecular Mechanisms for Repair of DNA (P.C. Hanawalt and R.B. Setlow, eds.; A. Hollaender, series ed.), Part A., Plenum Press, New York, 13-24.
- Sun, L., and Singer, B., (1975) The specificity of different classes of ethylating agents toward various sites of Hela cell DNA in vitro and in vivo, Biochem. 14:1795-1802.
- Tessman, I., Poddar, R.K., and Kumar, S., (1964) Identification of altered bases in mutated single-stranded DNA, I. In vitro mutagenesis by hydroxylamine, ethyl methanesulfonate and nitrous acid, J. Mol. Biol. 9:352-363.
- Verly, W.G., (1974) Monofunctional alkylating agents and apurinic sites in DNA, Biochem. Pharmacol. 23:3-8.
- Verly, W.G., (1975) Maintenance of DNA and repair of apurinic sites, in: Molecular Mechanisms for the Repair of DNA (P.C. Hanawalt and R.B. Setlow, eds.; A. Hollaender, series ed.), Part A, Plenum Press, New York, 39-46.
- Verly, W.G., Gossard, F., and Crine, P., (1974) In vitro repair of apurinic sites in DNA, Proc. Nat. Acad. Sci. U.S. 71:2273-2275.
- Verly, W.G., and Paquette, Y., (1973) An endonuclease for depurinated DNA in rat liver, Can. J. Biochem. 51:1003-1009.
- Verly, W.G., Paquette, Y., and Thibodeau, L., (1973) Nuclease for DNA apurinic sites may be involved in the maintenance of DNA in normal cells, Nature New Biology 244:67-69.
- Verly, W.G., and Rassart, E., (1975) Purification of Escherichia coli endonuclease specific for apurinic sites in DNA, J. Biol. Chem. 250:8214-8219.
- Witkin, E.M., (1975) Relationships among repair, mutagenesis and survival; Overview, in: Molecular Mechanisms for Repair of DNA (P.C. Hanawalt and R.B. Setlow, eds.; A. Hollaender, series ed.), Part A, Plenum Press, New York, 347-353.

Würgler, F.E., Ulrich, H., and Spring, H.W., (1968) An improved method of collecting large numbers of inseminated eggs of Drosophila melanogaster, Separatum Experimentia 24:1082-1083.

Yanofsky, C., Ito, J., and Horn, V., (1966) Amino acid replacement and the genetic code, Cold Spring Harbor Symp. Quant. Biol. 31:151-162.

APPENDIXES

APPENDIX I

TOTAL COUNTS, COUNTING TIME, % SPILLOVER, % EFFICIENCY FOR EXPERIMENTS ONE THROUGH SEVEN^a

Experiment	Treatment	No. of Eggs	³ H (A)	³² P (C)	Total Counting Time (min)	% SO ^b A--C	% SO C--A	% E ^c ³ H	% E ³² P
1 - Mate 1	Sperm	--	21463	57405	1200	0.16	5.5	21	81
	Embryos (180 min)	--	23619	106512	1200				
1 - Mate 2	Sperm	--	15924	31936	1200				
	Embryos (180 min)	--	21356	92609	1700				
2 - Mate 1	Sperm	--	10808	15598	1000	0.02	4.1	21	81
	Embryos (180 min)	--	16464	106765	1200				
	Embryos (3-12 hrs)	--	14231	88372	1100				
2 - Mate 2	Sperm	--	5278	10551	600				
	Embryos (180 min)	--	18254	134420	1200				
	Embryos (3-12 hrs)	--	16499	110753	1100				

APPENDIX I (Continued)

Experiment	Treatment	No. of Eggs	^3H (A)	^{32}P (C)	Total Counting Time (min)	% SO ^b A--C	% SO C--A	% E ^c ^3H	% E ^{32}P
3 - Feed 1 Mate 1	Sperm	--	17016	18616	2400	0.0015	0.83	20	68
	Embryos (20 min)	1116	10119	19568	1500				
	Embryos (180 min)	1128	10395	21732	1400				
	Embryos (3-12 hrs)	--	10815	36348	1300				
3 - Feed 2 Mate 1	Sperm	--	15370	16495	2000	0.0015	0.83	20	68
	Embryos (20 min)	2243	21730	57216	2486				
	Embryos (180 min)	2536	10022	32097	1100				
	Embryos (3-12 hrs)	--	10897	18634	1500				
4 - Mate 1	Sperm	--	28495	15223	1950				
	Embryos (15 min)	1478	16600	12448	1200				

APPENDIX I (Continued)

Experiment	Treatment	No. of Eggs	^3H (A)	^{32}P (C)	Total Counting Time (min)	% SO ^b A--C	% SO C--A	% E ^c ^3H	% E ^{32}P
4 - Mate 2	Embryos (25 min)	1386	18167	13764	1300				
	Embryos (180 min)	1842	12882	13474	800				
	Sperm	--	41395	26931	2850				
	Embryos (15 min)	2197	15156	24071	1550				
	Embryos (25 min)	2240	16920	31585	1680				
	Embryos (180 min)	2552	12608	24937	1250				
5 - Mate 1	Sperm	--	11524	12464	1680	0.0018	0.48	18	56
	Embryos (15 min)	3737	13228	20363	1873				
	Embryos (25 min)	3845	10650	18046	1500				
	Embryos (180 min)	2731	10273	16855	1544				

APPENDIX I (Continued)

Experiment	Treatment	No. of Eggs	^3H (A)	^{32}P (C)	Total Counting Time (min)	% SO ^b A--C	% SO C--A	% E ^c ^3H	% E ^{32}P
5 - Mate 2	Sperm	--	11385	13961	1452	0.0018	0.48	18	56
	Embryos (25 min)	2552	10926	19807	1700				
	Embryos (180 min)	2227	10715	20237	1642				
6 - Mate 1 Day 1	Sperm	--	18743	10449	1900	0.001	0.61	20	59
	Embryos (15 min)	1143	12309	10305	1400				
	Embryos (30 min)	1180	13813	11952	1500				
	Embryos (180 min)	1205	13553	13044	1500				
6 - Mate 1 Day 2	Sperm	--	14019	9598	1200				
	Embryos (15 min)	1220	9225	11501	1124				
	Embryos (30 min)	2030	11526	13400	1400				
	Embryos (180 min)	1305	10726	13221	1300				

APPENDIX I (Continued)

Experiment	Treatment	No. of Eggs	³ H (A)	³² P (C)	Total Counting Time (min)	% SO ^b A--C	% SO C--A	% E ^c ³ H	% E ³² P
6 - Mate 1 Day 3	Sperm	--	12858	9574	1200				
	Embryos (15 min)	743	13927	10921	1854				
	Embryos (30 min)	678	13161	11052	1761				
	Embryos (180 min)	730	13250	11284	1762				
6 - Mate 2 Day 1	Sperm	--	17724	10269	1500	0.001	0.61	20	59
6 - Mate 2 Day 2	Sperm	--	17472	10457	1500				
6 - Mate 2 Day 3	Sperm	--	15202	9382	1200				
<hr/>									
7	Sperm (0 hr MCE ^d)	--	59356	53817	300	0.001	0.60	20	65
	Sperm (1 hr MCE)	--	95568	80418	300				

APPENDIX I (Continued)

Experiment	Treatment	No. of Eggs	^3H (A)	^{32}P (C)	Total Counting Time (min)	% SO ^b A--C	% SO C--A	% E ^c ^3H	% E ^{32}P
	Sperm (4 hr MCE)	--	133112	112267	300				
	Embryos (30 min 0 hr MCE)	--	45922	13699	800				
	Embryos (30 min 1 hr MCE)	--	44000	13638	600				
	Embryos (30 min 4 hr MCE)	--	75909	22726	600				

^a Spillover and efficiency values are the same for each sample in a given experiment and are presented only once with the data from each experiment.

^b SO stands for spillover of counts in one counting channel to the other channel.

^c E stands for counting efficiency for a particular isotope.

^d MCE stands for mercaptoethanol.

APPENDIX II

TOTAL COUNTS AND COUNTING TIME
OF BLANKS FOR EXPERIMENTS ONE THROUGH SEVEN

Experiment	Blank	^3H (A)	^{32}P (C)	Time (min)
1	Sperm DNA	8198	9400	1100
	Embryos DNA	17830	16776	2100
2	Sperm DNA	9109	10459	1200
3	Sperm DNA	14284	16406	2285
	Embryos DNA	12993	15186	2100
4	Sperm DNA	14438	12163	1898
	Embryos DNA	23223	19583	3052
5	Sperm DNA	11508	11630	1800
	Embryos DNA	10849	11639	1800
6	Sperm DNA	17422	10704	2400
7	Sperm DNA	22966	11285	2600
	Embryos DNA	23491	11347	2600

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Dissertation contains pages with small and indistinct print. Filmed as received.

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APPENDIX III

PUBLICATIONS OF FRANK CHARLES JANCA

- Ficsor, G., Janca, F.C., and Van Hook, C. (1970) Tests for mutagenicity using Nitrosoguanidine, DDT and captan in maize, Newsletter of the Environmental Mutagen Society, No. 3:38 (Abstract).
- Ficsor, G., Beyer, R.D., Janca, F.C., and Zimmer, D.M. (1971) An organ-specific host-mediated microbial assay for detecting chemical mutagens in vivo: Demonstration of mutagenic action in rat testes following streptozotocin treatment, Mutation Res., 13:283-287.
- Janca, F.C. (1972) N-methyl-n'-nitro-N-nitrosoguanidine induced dominant and recessive mutations in Zea mays, Masters Thesis, Graduate College, Western Michigan University, Kalamazoo, Mi.
- Aaron, C.S., Lee, W.R., Janca, F.C. and Seamster, P.M. (1973) Ethylation of DNA in the germ line of male Drosophila melanogaster, Proceedings of the Thirteenth International Congress of Genetics, Berkeley, California, Genetics, 74:1s (Abstract).
- Lee, W.R., Aaron, C.S., and Janca, F.C. (1973) Dependence of alkylation and mutagenesis on germ cell stage, First International Conference on Environmental Mutagens, Asilomar, Pacific Grove, California (U.S.A.), Mutation Res., 21:226 (Abstract).
- *Aaron, C.S., Lee, W.R., Seamster, P.M. and Janca, F.C. (1977) A non-aqueous gas exposure technique for microliter quantities of chemical mutagens, Drosophila Information Service, 52:174-175.
- Janca, F.C., Lee, W.R., and Aaron, C.S., (1977) Stability of induced DNA alkylations in sperm and embryos of Drosophila melanogaster. Eighth Annual Meeting of the Environmental Mutagen Society, Colorado Springs, Colorado. I presented this paper at the Annual Meeting of the Environmental Mutagen Society.
- Lee, W.R., Aaron, C.S., Janca, F.C., and Skinner, P.M., (1977) Linear relation of alkylation to mutation in Drosophila. Second International Conference on Environmental Mutagens, Edinburgh, Scotland, Abstract Book p. 105.

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Aaron, C.S., W.R. Lee, P.M. Seamster and F. Janca. Louisiana State University, Baton Rouge. A non-aqueous gas exposure technique for microliter quantities of chemical mutagens.

tities of the mutagen is advantageous with specifically radiolabeled mutagens because of the expense of these compounds.

Vacuum injection of flies was previously described using aqueous solutions (Sega and Lee, DIS 45:179) but for reactive mutagens an aqueous solution may be undesirable. Furthermore, the maximum exposure concentration of a compound in the aqueous based vacuum injection procedure is limited to the compound's solubility in water times the maximum concentration of water that can be achieved in the treatment flask (limited by the dewpoint). It is more desirable to use true vapor of the mutagen if it is sufficiently volatile; therefore, we have developed a non-aqueous method of vacuum injection using glass fiber filter discs impregnated with the mutagen. For instance, when using ethyl methanesulfonate (EMS), we dissolve the mutagen in ethyl ether, place the filter disc in the ether at the bottom of a shell vial and carefully evaporate the ether in a stream of dry nitrogen thereby coating the glass filter with mutagen. We then place this filter in the vacuum release line of a special treatment apparatus (described here), thus entraining the mutagen in the air which fills the apparatus. (NOTE: Due to the extreme toxicity of ether to flies, particularly under vacuum injection conditions, it is imperative that no ether remain on the filter.)

The usual procedures for gases and aerosol treatment of *Drosophila melanogaster* with chemical mutagens require a volume of the mutagen as large as a milliliter or more. This note reports a method which we find to be adaptable for use with only microliter quantities of mutagens. The use of very small quantities

The apparatus which we have developed is diagrammed in Figure 1. The equipment used consists of a treatment vessel, high vacuum seal valves, a sintered glass funnel, and O-ring seal joints as well as necessary traps and connections to vacuum pump, air line, etc. The

Figure 1. Vacuum injection apparatus. 1) Valve #1; 2) Valve #2; 3) Air or N₂ inlet; 4) Vacuum line connection; 5) Air or N₂ outlet (to appropriate traps); 6) Sintered glass support funnel; 7) Glass fiber filter disc, impregnated with mutagen; 8) Septum for obtaining gas samples for gas chromatographic analysis. All joints (e.g., 9) are "O" ring real; dimensions are selected to present minimum area of contact between vapor and potentially absorbent materials. Either 3 or 4 may be connected to 2, and likewise either 5 or 6 may be connected to 1. Operation is described in the text.

treatment vessel is a modified 250 ml, 3 necked round bottomed flask. One neck was removed and replaced with a septum holder for collecting samples for gas chromatography. The other 2 necks have O-ring seal joints. The high vacuum seal valves (Kontes glass K-826515-0008 Kel-f (Pyrex No. 416320, 15 mm Medium grain) is fitted with an O-ring seal joint.

At the start of an experiment the flies are anesthetized with N₂ and shaken into the treatment vessel. The mutagen is impregnated into a small round piece (the standard size 14 corkborer is a satisfactory diameter) of glass fiber filter (Gelman Type A, # 61701) and placed on the sintered glass funnel which is mounted on the port of the #1 valve. Then, with #1 valve closed, a vacuum is pulled on the vessel (~10 mm Hg) using Valve #2. Valve #2 is closed and by opening Valve #1 the vacuum is released through the funnel, thus evaporating

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and entraining the chemical in the air stream. Valve #1 is again closed and the exposure is continued for a period of time (2-8 hrs). At the end of the treatment time, compressed air is admitted through Valve #2, out Valve #1 and subsequently through traps to flush out the system and destroy any remaining mutagen. Nitrogen flow in the same path (into Valve #2, through the flask, out Valve #1) is used to anesthetize the flies for removal from the vessel.

In view of the extremely hazardous nature of radiolabeled mutagens, great care must be taken to ensure the safety of laboratory personnel. The entire apparatus previously described should be contained within a glove box or fume hood. Alkaline mercaptoacetate solution (Lewis and Bacher, DIS 43:193) is used as a chemical trap for EMS in our laboratory. In addition, molecular sieve and cold traps are necessary to avoid radiochemical contamination of other equipment such as the vacuum pump.

The method described here has been used to induce a 6% sex-linked recessive lethal frequency by treating 50 ♂ with 2.0 mg of EMS for 8 hours. In a separate experiment 0.2 mg of radiolabeled EMS was administered to 1,000 males with significant alkylation of DNA observed (Janca, F.C., W.R. Lee and G.S. Aaron, unpublished).

Due to the variability in impregnation of the filter disc with the mutagen, reproducibility of this method of administering mutagens is not sufficient for constructing dosage response curves; however, this is the most economical method we have for treating a large number of males with small quantities of radiolabeled mutagens.

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VITA

Frank Charles Janca was born on October 27, 1946 in Oak Park, Illinois and is the eldest of three children born to August and Ella Janca. Shortly after his birth his parents purchased a farm and moved to the rural community of Decatur in south western Michigan.

His early elementary schooling from Kindergarten to sixth grade was spent in a one-room country school (East Decatur Public School No. 5) where he and the other neighborhood children were loved and taught by one of the most marvelous educators, Miss Mabel Schunaman. He attended Junior High School at Decatur Consolidated Public Schools and in June 1964 was among the first class to graduate from the newly constructed Decatur Jr-Sr High School.

In September of 1964 he entered Western Michigan University in Kalamazoo, Michigan and in 1968 he was graduated from that Institution with the Degree of Bachelor of Arts. During the fall of 1968 he was admitted to the Graduate Program in the Department of Zoology at WMU where he worked under the tutelage of Dr. Gyula Ficsor. In December of 1972 he received his Master of Arts Degree from WMU for work involving the investigation of the mutagenicity of N-methyl-N'-nitro-N-nitroso-guanidine in Zea mays. He came to Louisiana State University in the fall of 1971 to continue his graduate studies in genetics and mutagenesis under the guidance of Dr. William R. Lee. While attending Graduate School he gained valuable teaching and research experience by working as a teaching assistant and research assistant at WMU and LSU.

The work presented here is in partial fulfillment for the Degree of Doctor of Philosophy in Zoology and was completed by May 1978.

EXAMINATION AND THESIS REPORT

Candidate: Frank Charles Janca

Major Field: Zoology

Title of Thesis: Stability of induced DNA alkylations in sperm and embryos of Drosophila melanogaster.

Approved:

Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

Date of Examination: