Acta Biochimica Polonica

Vol. 45 No. 2/1998

545–559

QUARTERLY

Germ cell mutagenesis in Drosophila: Multiple endpoint analysis*°

Madeleine J.M. Nivard✉, John Wijen and Ekkehart W. Vogel

Department of Radiation Genetics and Chemical Mutagenesis, Medical Genetics Centre South-West Netherlands (MGC), University of Leiden, Wassenaarseweg 72, 2333 AL Leiden, Netherlands

Key words: Drosophila, mutagens, carcinogens, nucleotide excision repair, mutation spectra, clastogenicity, spermatogenesis

Genotoxic carcinogens, able to damage DNA by alkylation reactions, represent a very diverse class of agents which are capable of producing a wide range of DNA modifications. The mechanisms leading to genetic changes as a result of exposure to alkylating agents (AAs) have been studied in male germ cells of Drosophila using a structure-activity relationship approach (SAR). The analytical tools available concern both genetic and molecular assays. The genetic tests enable to quantify excision repair and clastogenic potency of the AA after treatment of post-meiotic male germ cells and to determine the degree of germ-cell specificity, i.e., the mutagenic effectiveness in post-*versus* premeiotic cell stages. For a selected group of alkylating agents the molecular spectra have been studied in post-meiotic cell stages. On the basis of these descriptors clear SAR's between genotoxic activity in germ cells and physico-chemical parameters (*s*-values and O^6/*N*7-alkylguanine adducts) and carcinogenic potency in rodents became apparent, resulting in five distinct classes of alkylating agents so far. These classes are: 1) S_N2-type monofunctional AAs, 2) S_N1-type monofunctional AAs, 3) polyfunctional AAs, 4) agents able to form etheno-DNA adducts, and 5) aflatoxin B1 (AFB1) a bulky-adduct forming agent.

The recent finding that the molecular data obtained with Drosophila and data of the specific locus tests in male mice show remarkable similarities for most genotoxic agents supports the view that Drosophila is a useful model system for the study of transgenerational damage.

---

*Paper presented at the Conference on "Mechanisms of DNA Repair and Mutagenesis" Commemorating the 100th Anniversary of the Discovery of Polonium and Radium, October, 1997, Warsaw, Poland.

°This work was supported by EEC programme Science and Technology for Environmental Protection (STEP), contract CT91-0145 and the Programme "Environment" of the European Commision, contract EV5V-CT94-0409.

✉Phone: (31) 71-527 6145; fax: (31) 71-522 1615; e-mail: nivard@rullf2.MedFac.LeidenUniv.nl

Abbreviations: AAs, alkylating agents; AFB1, aflatoxin B1; CEO, chlorethylene oxide; CL, loss of ring X-chromosome; DCE 1,2-dichloroethane; ECar, ethyl carbamate; CI, clastogenicity index; NER, nucleotide excision repair; 7OEG, 7(2-oxoethyl)-guanine; RL, recessive lethal; SAR, Structure Activity Relationship; VBr, vinyl bromide; VCl, vinyl chloride; and see Table 1.

The risk to develop certain types of cancers (breast and colon cancer) is related to 'inter-family' differences. The reason for this elevated risk in certain families to develop cancer is most likely related to the constitution of their genetic material (cancer prone families; Friend *et al.*, 1986; Malkin *et al.*, 1990; reviewed by Tomatis *et al.*, 1992). It is therefore important to understand not only the mechanisms of DNA damage formation by genotoxic carcinogens in somatic cells, but also the conditions for their genotoxic action at distinct cell-stages of the germline. The application of Drosophila as a useful model system to study germ cell mutagenesis is obvious when the following points are considered: (i) except for the length of the time period, the development of germ cells in Drosophila is quite similar to that in higher eukaryotes including man; (ii) studies with Drosophila are much cheaper, faster and therefore much less limited than studies on mammals; (iii) transgenic mutation assays (lac I, Big Blue^{TM} and *LacZ*, Muta^{TM} Mouse) are unable to analyse large chromosomal aberrations; (iv) there is no alternative *in vitro* system. Apart from the importance of studying risk analysis of mutagenesis at various germ cell stages, these cells are an excellent tool for Structure Activity Relationship (SAR) studies. The principle of SAR studies is that mutagens are divided in distinct classes based upon DNA damage profile, mutagenic activity and carcinogenic potency. The ultimate goal is to come up with a limited number of classes of mutagens, each representing a specific mechanism explaining the biological activity of the chemicals belonging to the same class and covering "all" genotoxins. The optimal application of SAR studies should include dosimetry experiments, which would then allow to quantify the various DNA adducts formed and to compare adduct levels with biological effects. However, the problem of dosimetry studies is that methods for quantification are available only for a limited number of DNA adducts. Semi-quantitative approaches in which induction of various mutagenic endpoints are compared with each other seem to be an acceptable alternative. Studies have indicated that many chemicals induce both gene and chromosomal mutations. The essential of this approach is that the recovery and analysis of the various mutagenic endpoints are determined in the same cell type, such as the postmeiotic male germ cells of Drosophila.

The various biological endpoints used in the multiple endpoint analysis are: (i) the response in a DNA repair assay, using excision repair deficient mutants; *mus201* (Boyd *et al.*, 1976; 1982) or *mei-9* (Graf *et al.*, 1979); (ii) determination of the clastogenic potency by estimating the ratio of induced ring-X losses and forward mutations (Zijlstra & Vogel, 1988); (iii) the molecular spectra of mutations induced in a specific locus (*vermilion* gene located on the X-chromosome). Also included in these multiple endpoint analysis is the activity of mutagens in post- *versus* premeiotic stages of male germ cells. A characteristic feature of postmeiotic male germ cells is the absence of active DNA repair (Lee & Kelly, 1986; Smith *et al.*, 1983). The consequence is that Drosophila spermatozoa and spermatid stages are susceptible to all kinds of DNA alkyl adducts leading to mutations. Premeiotic cell stages are less susceptible to mutagenic effects due to efficient repair of DNA damage. Furthermore, germinal selection may eliminate certain types of mutations (Shukla & Auerbach, 1980; Vogel *et al.*, 1982). In terms of genetic risks we have addressed the question whether chemicals having low TD_{50} values are also those producing mutagenic activities in a wide range of germ cells.

Data on mutagenic activity in Drosophila germ cells are evaluated against the features of nucleophilic selectivity (Swain Scott's constant *s*), DNA-adduct patterns and overall carcinogenicity data in rodents, in order to obtain a risk-analysis based on genetic data obtained in Drosophila. To avoid overlap with recently published work the paper is rather short. However, it contains a number of key

references giving the reader access to our present research on SAR concepts.

MATERIAL AND METHODS

Development of Drosophila germ cells

The adult testes of Drosophila contain germ cells at distinct stages of development ranging from undifferentiated spermatogonia to mature spermatozoa. It takes spermatogonia stem cells about 5 days to pass through the various premeiotic stages before they enter the two meiotic divisions. The subsequent development of postmeiotic cells into mature spermatozoa lasts approximately 4–5 days (Lindsley & Tokuyasu, 1980). The postmeiotic cell stage is unique in the sense that the cells are haploid and the later stages have no active DNA repair (Lee & Kelley, 1986; Smith *et al.*, 1983).

Thus, efficient repair of premutational damage induced in male germ cells takes place at early stages of the germ cells and in the egg after fertilization; late spermatids and spermatozoa have lost their capability of repair. This property is a general characteristic of both Drosophila and mammalian systems including man (Sega, 1990).

Exposure techniques

Adult male flies were exposed in all experiments. The different properties and stabilities of the test substances required the use of different application techniques. The most general method which can be used if chemicals are stable, is adult feeding. Males are placed in vials containing 8 layers of glass microfiber paper soaked with 0.9 ml of a mutagen solution containing 5% (w/v) sucrose. In the case of highly reactive chemicals, the only possibility of treatment is injection into the abdomen. The third class of mutagens are those which are volatile, as for instance vinyl bromide (VBr). The appropriate method of treatment is then by inhalation. Flies are put in a 530 ml bottle containing on the bottom 8 layers of glass microfiber paper soaked with 9 ml buffer containing 5% (w/v) sucrose. After injection of the liquid mutagen the bottle is tightly closed.

Genetic assays

Measurement of recessive lethal mutations. The principle of the recessive lethal test is based on the fact that genetic changes in hemizygous and homozygous, but not in heterozygous, conditions are lethal for the developing fly. The changes must be induced in genes called recessive lethal genes. Recesssive lethal mutation induction has been determined both for the X-chromosome and chromosome 2. The number of recessive lethal genes on chromosome 2 is twice the number of genes located on the X-chromosome, i.e. 1400 on chromosome 2 and 700 on the X-chromosome (Abrahamson *et al.*, 1980). Therefore, a 2-fold higher mutation rate would be expected for chromosome 2 compared to the X-chromosome ($M_{chrom\ 2}/M_{X\text{-}chrom} = 2$).

DNA repair assay for hypermutability response. The DNA repair assay is set up in such a way that sex-linked recessive lethal mutant frequencies induced under proficient or deficient DNA repair conditions are compared, by crossing exposed males with either nucleotide excision repair deficient (NER^-) or proficient (NER^+) virgins until five days after treatment. In this way only mutations in mature sperm cells and late spermatids are measured, i.e., at cell stages which have no active DNA repair. The resulting M_{NER^-} / M_{NER^+} hypermutability index provides information on the efficiency of *in vivo* DNA repair in the oocyte in relation to the type of inducing agent (Vogel, 1989).

Comparison of mutagenic activity in post- and premeiotic cell stages. To study mutagenic activity at post and premeiotic cell stages, the exposed males are mated every three or four days with a new set of virgins.

The progeny of the first two or three broods (postmeiotic male germ cells) and of broods 5 and 6 (premeiotic male germ cells) were tested for increased mutation frequencies.

Induction of loss of a ring-shaped X-chromosome. The assay is based on the fact that loss of a ring-shaped X-chromosome in the progeny of treated males gives flies with a distinct phenotype. Use of flies carrying a ring-shaped instead of a rod-shaped X-chromosome strongly enhances both the spontaneous and mutagen induced frequencies of X-chromosome loss. The events leading to loss of the ring X-chromosome are generally believed to result from chromosome breakage or sister chromatid exchanges, although the latter events are rare in Drosophila (Gatti, 1982).

Clastogenic efficiency. This analysis compares the induction of loss of ring X-chromosome (CL) with the induction of forward mutations (RL), after exposure of postmeiotic male germ cells to a mutagen. The CL/RL ratio or clastogenicity index $I_{CL/RL}$ obtained by this procedure has a prognostic value in terms of the number of functional groups of genotoxic chemicals. It was shown that the assay discriminates between monofunctional agents (ratios < 1) and agents capable of cross-linking DNA (ratios > 2) (Zijlstra & Vogel, 1988).

Molecular analysis: the *vermilion* locus

Mutations are induced in the *vermilion* locus which is a recessive gene located on the X-chromosome of Drosophila. Mutant flies can be selected by a phenotypic change in the eye colour of the mutant. The size of the gene is only 2 kb. Using PCR techniques and sequence analysis, point mutations, frame shift mutations, small intra-locus deletions and insertions can be analysed. Furthermore, the location of the *vermilion* gene is such that proximal to the gene is located a haplo-insufficient fertility gene, and distal to it a recessive lethal gene. *Vermilion* mutants that are either heterozygous sterile or hemizygous lethal may carry a multi-locus deletion covering the *vermilion* gene and one of the relevant proximal or distal genes. Molecular and cytogenetic techniques are available to analyse also these type of mutations.

RESULTS AND DISCUSSION

For more than 20 years germ cells of Drosophila have been used to study SAR concepts. At the beginning mainly methylating and ethylating agents were investigated, but this research has been expanded to a large variety of mutagens. Table 1 lists those mutagens studied in postmeiotic male germ cells of Drosophila for which both genetic and molecular data are available. The mutagens are divided in distinct classes based on their DNA adduct formation profile, carcinogenic potency and the outcome of the multiple endpoint analysis in Drosophila germ cells. The genetic studies include two assays. The first assay quantifies mutation induction under repair deficient and proficient conditions (M_{NER^-} / M_{NER^+}), in order to determine information on the efficiency of the DNA repair system against induced DNA damage (Vogel, 1989). The second assay determines the clastogenic potency of mutagens, by comparing the induction of chromosomal loss events with the induction of forward mutations ($I_{CL/RL}$). The $I_{CL/RL}$ index has been measured for more than 45 mutagens (Vogel *et al.*, 1993). The outcome of this evaluation was that the $I_{CL/RL}$ index had a prognostic value, i.e., the index indicates whether mutagens have either monofunctional or polyfunctional properties. Molecular data are obtained from a specific locus test in which mutations were induced in the *vermilion* gene located on the X-chromosome. DNA of the *vermilion* mutants were isolated and the mutations analysed.

In the next section the results of the three different endpoints measured in Drosophila germ cells will be discussed for each class of

Table 1. Genetic and molecular activity profiles of distinct classes of alkylating agents in Drosophila in comparison with median TD_{50} values for rodents.

Mutagen	s-Value[a]	TD_{50} in rodents[b] (mmol/kg b.wt.	M_{NER^-}/M_{NER^+}[c]	ICL/RL[d]	Molecular analysis[e]		
					bpc	Del(ild/mld)	References
Methylating and ethylating agents							
Methyl methanesulphonate (MMS)	0.83	11.27	8.2	0.55	45%	55% (23/32)	(1,4)
N-Methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG)	0.42	0.183	3.6	0.19	30%	70% (35/35)	(4)
N-Nitrosodimethylamine (DMN)		1.88	6.4	0.16	56%	44% (20/24)	(4)
N-Nitroso-*N*-methylurea (MNU)	0.42	0.271	5.8	0.23	52%	48% (32/16)	(4)
Diethyl sulphate (DES)	0.64	(0.648)	2.5	0.13	100%		(2)
Ethyl methanesulphonate (EMS)	0.67	(6.04)	2.7	0.32	89%	11%	(3)
N-Nitrosodiethylamine (DEN)		0.549	1.3	CL neg	88%	12%	(5#)
N-Nitroso-*N*-ethylurea (ENU)	0.26	0.0939	1.3	0.04	91%	9%	(6#)
Agents forming substituted ethyl-DNA adducts							
1,2-Dibromoethane (DBE)	(1.2)[aa]	8.78	7.2	0.37	0%	100% (78/22)	(12)
2-Chloroethylamine (CEA)			(20)	(1-2)	no data*		
Ethylene oxide (EO)	0.96	229	12	1.5	no data*		
Polyfunctional alkylating agents							
1,3-Bis-(2-chloroethyl)-1-nitrosourea (BCNU)	0.94	0.159	1.5	4.3	8%	92% (33/58)	(7)
Chlorambucil (CAB)	1.26	0.302	(2.4)	3.3	Under test		(8)
Cisplatin (DDP)		0.0667	NT	6.8	Mainly intra and multi-locus deletions		(9)
Hexamethylmelamine (HMM)			NT	8.6	6%	94% (29/65)	(10)
Hexamethylphosphoramide (HMPA)		0.151	0.8	12.0	0%	100% (37/63)	(11)
Mechlorethamine (MEC)	1.18	0.088	(3.5)	4.1	14%	86% (43/43)	(8)
Other alkylating agents							
forming of etheno-DNA adducts							
Vinyl bromide (VBr)		125	4.5	2.7	no data*		
Vinyl carbamate (VCar)		(3)	5.8	6.9	31%	69% (25/44)	(13)
cyclic agents							
1,3-Propane sultone (PS)	0.71	14.4	4.9	0.08	75%	25% (17/8)	(7)
β-Propiolactone (β-PL)	0.77	1.94	6.3	0.39	40%	60% (30/30)	(7)
bulky-adduct forming agent							
Aflatoxin B1		0.0145	(2.0)	(0.5)	50%	50% (8/42)	(7)

Legend to Table 1 on the next page.

mutagens and evaluated against, if available, *s*-values or DNA adduct patterns and TD_{50} values from rodents (see Table 1).

Methylating and ethylating agents with high and low *s* values

The Swain-Scott *s* value is a measure for the nucleophilic selectivity of the alkylating agent (Swain & Scott, 1953). This means that alkylating agents with high *s* values have a strong preference for reacting with centres of high nucleophilicity. In DNA, nitrogens are the stronger nucleophilic positions compared to the oxygens. Quantitative adduct measurements showed for several alkylating agents a clear correlation between $N7/O^6$-alkylguanine ratios and *s* values (Beranek, 1990). The overall DNA adduct pattern determines not only the mutagenic activity of these agents but also the magnitude of the mutational response seen in germ cells with a defective DNA excision repair mechanism. A clear relation exists between *s* values, or $N7/O^6$-alkylguanine ratios, and enhanced mutagenic activity in NER^- compared to repair proficient conditions. This hypermutability effect is more pronounced for methylating agents than for the ethyl structural analogues.

Molecular analyses of mutants induced at postmeiotic cell stages showed that ethylating agents induce mainly base pair substitutions generated by DNA adducts on oxygen positions: O^6-ethylguanine causing GC→AT transitions and O^4-ethylthymine causing AT→GC transitions. Ethyl methanesulfonate (EMS) and diethyl sulphate (DES), high *s* mutagens, induced more frequent GC→AT transitions, 76% and 73%, respectively, than the lower *s* agents *N*-nitroso-*N*-ethylurea (ENU) (61%) and *N*-nitrosodiethylamine (DEN) (39%) (for references see Table 1). These last two chemicals, in contrast to EMS and DES, ethylate oxygen positions of pyrimidines in a measurable amount (Beranek, 1990). The spectra of methylating agents including those with low *s* values showed high percentages of deletions and only few GC→AT transitions. Obviously alkyltransferases, which are present in Drosophila, remove methyl groups more efficiently than ethyl groups. The molecular spectra of low *s* methylating agents, like *N*-nitroso-*N*-methylurea (MNU), *N*-nitrosodimethylamine (DMN) and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), contained also significant numbers of AT→GC transitions suggesting that O^4-methylthymine is an important mutagenic lesion and that the alkyltransferase repairs this lesion less efficiently than O^6-methylguanine (Fig. 1).

The initial damage responsible for the formation of deletions and most transversions are probably nitrogen DNA adducts which are substrates for the excision repair mechanism. The relative contribution of deletions to the spectra can be predicted from the $I_{CL/RL}$ ratios which are for methylating agents higher than for ethylating agents.

A most interesting phenomenon in Drosophila is that the relative increase in mutation induction seen under repair deficient conditions increases with the time interval between

Legend to Table 1

[a] *s* values are categorized by Vogel *et al.* 1990. [aa]Mutagenic activity of DBE only after metabolic activation; [b] Barbin & Bartsch, 1989; Vogel *et al.* 1990. Values in parentheses are inaccurate or a preliminary estimate; [c] Ballering *et al.*, 1993, 1996; Vogel, 1989; Vogel & Nivard, 1997. Values in parentheses are preliminary, experiments are currently being repeated (own data); [d] Ballering *et al.*, 1996; Zijlstra & Vogel 1988, Vogel *et al.*, 1990; 1993. Values in parentheses are preliminary, experiments are currently being repeated (own data). [e]The molecular data are from F_1 complete mutants obtained in a repair proficient background. Data from F_1 mosaic mutants are in the references. [f]Sterile mutants were not analyzed which may have resulted in an underestimation of multi-locus deletions. [g] Specific locus mutant frequency under repair proficient conditions less than 10 times the spontaneous induction. (1) Nivard *et al.*, 1992; (2)Sierra *et al.*, 1993; (3) Pastink *et al.*, 1991; (4) Nivard *et al.*, 1996; (5) Sierra, unpublished; (6) Pastink *et al.*, 1989; (7) Nivard, unpublished; (8) Wijen (preliminary data); (9) Cizeau *et al.*, 1994; (10) Aquirrezabalaga *et al.*, 1995a; (11) Aquirrezabalaga *et al.*, 1995b; (12) Ballering *et al.*, 1994; (13) Ballering *et al.*, 1997. NT, not tested.

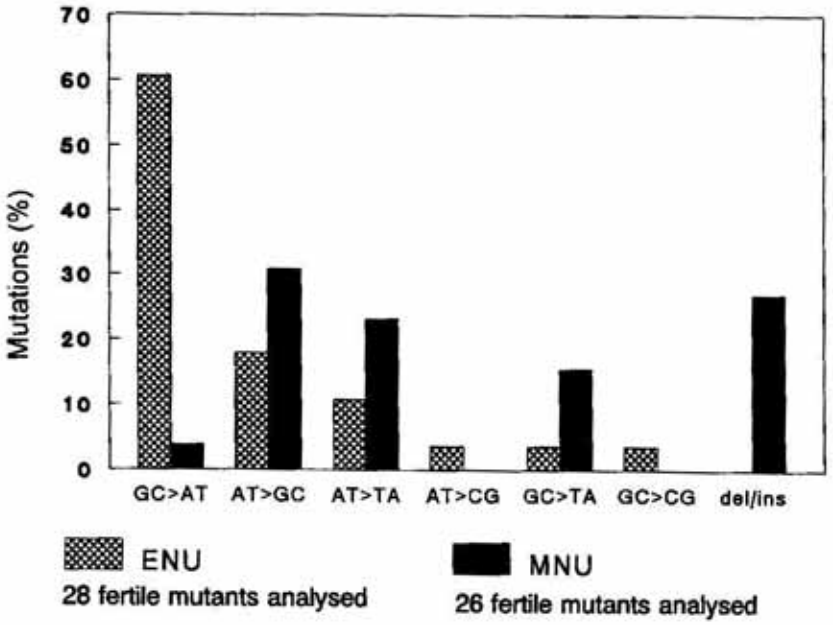

Figure 1. Percentages of distinct mutations analysed from fertile F_1 complete and mosaic mutants induced by ENU (Pastink *et al.*, 1989) and MNU (Nivard *et al.*, 1996) in Drosophila post-meiotic male germ cells.

Del/ins means intra-locus deletion and/or insertion.

adduct formation at postmeiotic cell stages and repair of DNA damage in the oocyte. In other words, the hypermutability effect for mutagenized late spermatids is higher than for spermatozoa (Vogel, 1989). This suggests that chemically unstable DNA adducts, like *N*3- and *N*7-alkylpurines forming apurinic sites, are responsible for the increased mutagenicity under NER^- condition. In fact the spectra of mutations induced by methyl methanesulfonate (MMS), MNU, DMN and EMS under repair deficient conditions showed a clear increase in the formation of transversions (Fig. 2), both AT→TA and GC→TA transversions, which confirmed the role of AP-sites for the increased mutability in the NER^- genotype (references see Table 1; Nivard *et al.*, 1993).

Concerning analysis of potential risk resulting from exposure to methylating and ethylating agents, there is an intriguing positive correlation between relative potency ranking of carcinogens (Bartsch *et al.*, 1983; Barbin & Bartsch, 1986; 1989), and relative efficiency

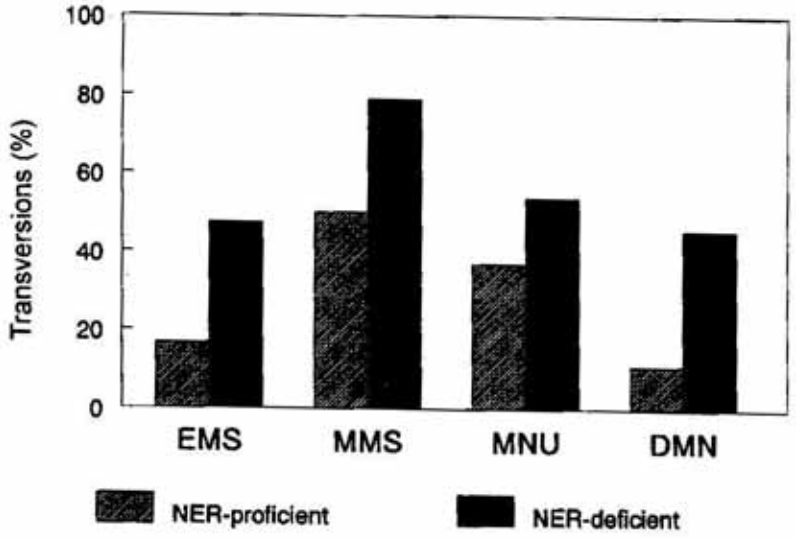

Figure 2. Percentages of transversions in the spectra of mutations obtained by EMS, MMS, MNU and DMN under repair proficient (NER^+) and deficient (NER^-) conditions.

Mutations were analysed from fertile F_1 complete mutants. Data are from literature: EMS (Pastink *et al.*, 1991); MMS (Nivard *et al.*, 1992; 1993), MNU and DMN (Nivard *et al.*, 1996).

Table 2. Mutant frequencies and ratios of mutant frequencies at post- and at pre-meiotic male germ cell stages of Drosophila

Mutagen	Mutant frequency X-chromosome[b]		Mutant frequency chromosome 2[b]		Ratio $M_{post}/M_{pre\text{-}meiotic}$		Ratio $M_{chrom2}/M_{X\text{-}chrom}$	
	post-meiotic	pre-meiotic	post-meiotic	pre-meiotic	X-chrom	Chrom 2	post-meiotic	pre-meiotic
High *s*-alkylating agent								
MMS[1]	6.25%	0.30%	24.85%	1.40%	30	21	4.0	8.0
EMS[2]	9.8%	0.53%	16.8%	4.9%	22	3.5	1.7	12.4
Low *s*-alkylating agent								
DEN[3]	2.3%	5.9%	5.6%	18.4%	0.4	0.3	2.5	3.2
ENU[1]	29.25%	8.37%	41.70%	19.59%	3.5	2.1	1.4	2.4
Poly-functional alkylating agent								
diepoxybutane DEB[3]	11.25%	2.16%	23.17%	18.89%	5.4	1.2	2.1	9.3
MEC[4]	2.06%	0.77%	5.5%	3.2%	2.7	1.8	2.2	> 21
BCNU[5]	9.5%	1.3%	ND	ND	8.1	-		
Etheno-adduct forming agent								
VCar[5]	6.3%	0.22%	2.6%	2.3%	88[a]	1.1[a]	-[a]	-[a]
VC1[6]	2.6%	0.89%	ND	ND	2.9	-		
ECar[7]	5.5%	1.23%	ND	ND	4.5	-		
Bulky-adduct forming agent								
AFB1[5]	2.6%	0.35%	2.2%	2.1%	12[a]	1.1[a]	-[a]	-[a]

[1]Vogel & Nivard, 1997; [2]Vogel *et al.*, 1982; [3]Shukla & Auerbach, 1980; [4]Wijen, unpublished; [5]Nivard, unpublished; [6]Verburgt & Vogel, 1977; [7]Nomura, 1979; [a]different exposure doses used in the experiments on mutation induction on X- and chromosome 2. [b]spontaneous mutant frequencies between 0.15 (X-chromosome) and 0.4 % (chromosome 2). ND: not determined.

ranking with respect to hypermutability in NER$^-$ strains in Drosophila. Furthermore, data on germ cells of Drosophila and the mouse (reviewed by Vogel & Natarajan, 1995) show that low *s* alkylating agents are strongly mutagenic at all stages of the spermatogenesis, including the DNA repair active premeiotic cell stages, whereas for the high *s* chemicals MMS and EMS the mutagenic activity is strongly reduced at premeiotic stages of Drosophila (Table 2). In the mouse they are only genotoxic at germ cell stages that have passed the meiotic divisions. Molecular data on ENU induced mutations in both Drosophila and mouse premeiotic cells showed that, in both organisms, mainly basepair changes on AT sites were induced whereas only few GC→AT transitions were found (Marker *et al.*, 1997; Vogel *et al.*, 1996).

In conclusion, the carcinogenic and mutagenic potency of monofunctional alkylating agents correlates with damage on oxygen positions in DNA, especially O^2- and O^4-alkylthymine adducts which are apparently poorly repaired. Damage on nitrogen positions in DNA is efficiently repaired and therefore weakly mutagenic. Formation of DNA adducts on nitrogens is also considered to be relevant for clastogenic effects.

Agents forming substituted ethyl-DNA adducts

Chemicals belonging to this group are ethylene oxide (EO), vinyl halides such as 1,2-dibromoethane (DBE) and the half-mustard 2-chloroethylamine (CEA). EO and CEA are direct acting monofunctional alkylating agents forming hydroxyethyl and aminoethyl DNA adducts, respectively. DBE is an indirect acting mutagen. Although both cytochrome P450 and glutathione may induce active metabolites (Dekant & Vamvakas, 1993), the general concept is that, for mutagenicity, conjugation with gluthatione is the most important route of metabolic conversion (Laib, 1986; Ballering *et al.*, 1993). All three agents showed a strong

increase in mutation induction in a NER⁻ genotype. TD_{50} values for both DBE and EO are high. These biological effects are consistent with the model that these agents react mainly on nitrogen positions in DNA, damage which is efficiently repaired by the excision repair mechanism. *In vitro* reactions of DNA with DBE and 1,2-dichloroethane (DCE) have shown that the major DNA adduct formed following glutathione activation is *S*-[2-(*N*7-guanyl)ethyl]glutathione (Koga *et al.*, 1986). The molecular spectra obtained after exposure of postmeiotic male germ cells to DBE contained only deletions when F1 complete mutants were studied, whereas F1 mosaic mutants and mutants isolated under NER⁻ conditions contained mainly base substitutions. Thirteen of these fourteen base pair changes were on GC positions suggesting a large contribution of *S*-[2-(*N*7-guanyl)ethyl]-glutathione adducts to the mutagenic activity of DBE, an adduct which is apparently efficiently removed by the NER system.

The nature of mutations induced by EO and CEA could be studied only with mutants derived from NER⁻ conditions due to the low induction level by these agents in wild-type flies. The EO spectrum contained 27% deletions. The base substitutions were both on AT and GC positions including transitions and transversions, which confirms literature data reporting that *N*7-hydroxyethylguanine and *N*3-hydroxyethyladenine, the two major adducts formed, are possibly involved in EO induced mutagenesis (Segerbäck, 1990).

In conclusion, agents giving substituted ethyl-DNA adducts behave like methylating and ethylating agents. The mutagens studied, EO, DBE and CEA, like high *s* alkylating agents, have a preference to react with the nitrogens in DNA. They are weak carcinogens due to efficient repair of the major DNA adducts formed. Agents of this type have been predicted to be very weakly active or even inactive at premeiotic male germ cell stages of Drosophila, as has also been found for EO in mouse spermatogonial stem-cells (Russell *et al.*, 1984).

Polyfunctional alkylating agents

The characteristic property of polyfunctional alkylating agents is that, apart from formation of mono-adducts with DNA, they may form intra- and interstrand DNA cross-links. Thus the question is which of these DNA adducts cause mutagenic and carcinogenic activity. Estimates of hypermutability indexes indicated that in general the DNA modifications responsible for mutagenic activity of cross-linking agents are not efficiently removed by the NER system (see also Vogel, 1989). With HMPA even a hypomutability was observed. This absence of a DNA repair effect for most polyfunctional agents, independent of their *s*-values, suggested that the most effective mutagenic lesions are not mono-adducts but rather DNA cross-links, thus a damage not well repaired by the NER system. Molecular analysis of mutations induced by five different polyfunctional agents, obtained from both complete as well as from mosaic mutants, showed almost exclusively intra- and multi-locus deletions. Similar results have been obtained from mutants induced by either chlorambucil or melphalan in post-spermatogonial mice germ cells: 24 of the 29 mutations were large deletions (Russell & Rinchik, 1993). Such a dominance of rearrangement mutations has not been found for any monofunctional agent. These data provide strong evidence that DNA crosslinks are responsible for the mutagenic activity of these agents. In this respect, mechlorethamine (MEC) is an interesting case because it showed a clear hypermutability effect ($M_{NER^-}/M_{NER^+} = 3.5$) and produced the highest contribution of point mutations to the molecular spectra. This suggests that in MEC-induced mutagenesis also mono-adducts are relevant for the mutagenic activity, especially if the NER system is not optimally functioning. We therefore antici-

pate that under repair deficient conditions the contribution of base pair changes to the molecular spectra will be more pronounced compared to the proficient conditions. This hypothesis is currently under investigation in our Institute. For hexamethylphosphoramide (HMPA) such a comparison has already been made but, as expected on basis of the M_{NER^-}/M_{NER^+} ratio of about 1, the spectra from deficient conditions did not show an increase in base pair changes (Aquirrezabalaga *et al.*, 1995b).

The high contribution of chromosomal aberrations to the molecular spectra of polyfunctional alkylating agents was also reflected in their high $I_{CL/RL}$ indexes, which were for all cross-linking agents higher than two. Such high ratios were not yet found for any monofunctional agent (Vogel *et al.*, 1993).

Some agents have been studied at premeiotic cell stages of Drosophila (Table 2). A preliminary outcome of these studies is that, although the mutagenic activity on the X-chromosome is strongly reduced compared to postmeiotic cell stages, autosomes seem to be highly mutable at premeiotic cell stages.

In summary, direct-acting polyfunctional agents are strong carcinogens, their TD_{50} values are all within one order of magnitude and do not correlate with *s*-values. These data, together with the outcome of the Drosophila experiments, provide evidence that not monoadducts but rather DNA cross-links are responsible for the mutagenic and carcinogenic activity of polyfunctional agents.

Other alkylating agents

Agents forming etheno-DNA adducts

Vinyl halides and related compounds such as ethyl carbamate (ECar) and vinyl carbamate (VCar), after conversion into their ultimate metabolites, give ethenoadducts with DNA. *In vivo* and *in vitro* reactions of DNA and RNA with several of these agents, including chlorethylene oxide (CEO), showed five DNA adducts: the major adduct being 7(2-oxoethyl)-guanine (7OEG) and 4 minor ethenoadducts: 1,N^6-ethenoadenine (εA), 3,N^4-ethenocytosine (εC), N^2,3-ethenoguanine (εG), and 1,N^2-ethenoguanine (1,N^2-εG). The latter adduct has not been detected *in vivo* so far. Data on the persistence of the various DNA adducts *in vivo* showed that 7OEG had a half life of 62 h whereas all three ethenoadducts were highly persistent (Bartsch *et al.*, 1994; Fedtke *et al.*, 1990).

Etheno DNA-adduct forming chemicals are bifunctional by definition, and thus may also form DNA cross-links. The $I_{CL/RL}$ ratios of these agents were all above two (see Ballering *et al.*, 1996), pointing to their polyfunctional properties. But, in contrast to the class of polyfunctional agents, clear hypermutability effects under NER deficient conditions have not only been measured for vinyl bromide (VBr) and VCar (Table 1) but also for vinyl chloride (VCl), ECar (Ballering *et al.*, 1996) and vinylcarbamate-epoxide (not published). CEO, however, the assumed ultimate metabolite of VCl, did not show a hypermutability effect (Vogel, 1989). This unexpected finding suggested that CEO is not the only metabolite of VCl.

The molecular spectrum of VCar mutations induced in a repair proficient background showed, in addition to deletions, also base pair changes at both AT and GC positions. The percentage of base pair changes in the VCar spectrum was much higher than in the spectra of cross-linking agents. This suggested that ethenoadducts are involved in the mutagenic activity of VCar. Under repair deficient conditions the contribution of base pair changes was further increased (50% point mutations). For VBr only mutants induced under repair deficient conditions could be studied, due to its low mutagenic effectiveness in the wild-type. The VBr spectrum contained 11 point mutations among the 14 mutations analysed.

In conclusion, although etheno-DNA adduct forming chemicals are bifunctional, it is unlikely that DNA cross-links are relevant for

their mutagenic activity. They form a separate class on their own because the combination of a high hypermutability index and a high clastogenic potency has not been observed for any other monofunctional or polyfunctional alkylating agent. This strongly suggests the involvement of etheno-adducts in their mutagenicity.

The mutagenic activity of ECar, VCar and VCl has been studied also at premeiotic germ cell stages. ECar and VCl were clearly mutagenic on the X-chromosome, although the activity was reduced compared to postmeiotic stages. In premeiotic cells of mutagenized males, mutagenicity of VCar was, like that observed for polyfunctional agents, clearly higher for chromosome 2 compared to the X-chromosome. The activity of etheno-DNA adduct forming mutagens at premeiotic stages was unexpected for two reasons. (i) The high hypermutability indexes suggested that DNA damage induced in premeiotic cells under conditions of active DNA repair would efficiently be repaired (like with MMS). (ii) We assume that carcinogenic potency is related with mutagenic activity displayed at all germ cell stages including premeiotic cell stages, but the TD_{50} values of these mutagens are not particularly low. However, it should be kept in mind that TD_{50} values are estimated from exposure doses with no correction for differences in metabolic activation processes. An explanation for the mutagenic activity in premeiotic cells may be that, in addition to DNA lesions which are efficiently repaired, they induce, although in minor amounts, some premutagenic lesions which are not or are only inefficiently repaired. The persistent etheno-adducts may be good candidates for this activity.

Cyclic agents

1,3-Propane sultone (PS) and β-propiolactone (β-PL) are two mutagens which also may form cyclic adducts with bases in the DNA. Again these agents are bi-functional by definition, but are probably different from the group of agents exerting their mutagenic activity through the formation of DNA cross-links. The $I_{CL/RL}$ index for both PS and β-PL is smaller than 1, indicating that they are weak clastogens clearly distinct from polyfunctional and etheno-adduct forming mutagens. This observation, together with the clearly enhanced mutation induction in NER deficient conditions, the contribution of both point mutations and deletions in their spectra, suggested that the genotoxicity of PS and β-PL follows similar mechanisms as that of small, monofunctional alkylating agents.

Agents forming bulky adducts with the DNA

Aflatoxin B1 is a potent liver carcinogen (IARC, 1976; Wogan, 1973). In the SLRL forward mutation test AFB1 expresses its activity in the early spermatids rather than in mature sperm cells, implicating that AFB1 must be metabolized and that the active metabolite is not or is only ineffectively transported to mature sperm cells. A striking observation was that induction of mutations calculated per mmol exposure was decreasing over a wide dose range from 0.001 mM to 0.3 mM (until now the lowest effective dose has not yet been reached, and experiments are still running). This suggests that the metabolism of AFB1 to its ultimate metabolite is a rate limiting step. Nevertheless, the high mutagenic activity based on exposition dose seems in agreement with strong carcinogenic potency of AFB1.

Lamb and Lilly (1971) also reported for chromosome II a weak mutagenic activity of AFB1 in mature sperm cells, but this activity clearly increased at earlier germ cell stages including premeiotic cells. Preliminary data on premeiotic activity on the X-chromosome have shown indeed positive results; again the activity seems to be higher for chromosome 2 than for the X-chromosome.

The spectrum of AFB1 mutations derived from postmeiotic cell stages contained 50%

point mutations which all except one were on GC positions: mainly GC→TA which is in agreement with the general idea that the *N*7-guanine adduct, 8,9-dihydro-8-(*N*7-guanyl)-9-hydroxy-AFB1, is the adduct responsible for mutagenic activity. The other 50% were deletions, mainly multi-locus deletions.

CONCLUSIONS

- Alkylating agents (AAs) are a most interesting class of compounds to investigate mutagenic action principles in germ cells. Genetic and molecular data obtained from studies on post-meiotic Drosophila male germ cells in combination with mutagenic activity at pre-meiotic cell stages, data on nucleophilic selectivity and carcinogenic potency in rodents, revealed so far five distinct classes of alkylating agents:
- **1.** S_N2-type monofunctional AAs are agents with high Swain Scott *s* values which predominantly react with ring nitrogens in DNA. These agents are weak mutagens and carcinogens probably because the nitrogen DNA adducts are well repaired. In agreement with efficient repair of DNA damage induction, the mutagenic activity at premeiotic cell stages is strongly reduced. Representatives of this class are MMS (methyl methanesulfonate) and EO (ethylene oxide).
- **2.** S_N1-type monofunctional AAs are agents with low *s* values. Alkylation of oxygen positions in DNA, especially O^4- and O^2-alkylthymine, determines their high mutagenic and carcinogenic potency. This group of agents are mutagenic at all germ cell stages. Examples are ENU (*N*-ethyl-*N*-nitrosourea), MNU (*N*-methyl-*N*-nitrosourea) and DMN (*N*-nitrosodimethylamine).
- **3.** Polyfunctional AAs are able to form cross-links with DNA. Examples are mechloroethamine (MEC), chlorambucil (CAB) and 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU). Their results obtained suggest that the formation of DNA crosslinks is responsible for their high carcinogenic and mutagenic potency. They all induce mainly deletions in postmeiotic male germ cells, which is in accordance with their strong clastogenic activity at these stages. For yet unknown reason this activity at premeiotic stages is much higher for autosomes than for the X-chromosome.
- **4.** Agents able to form etheno DNA-adducts. The combination of increased mutagenic activity under excision repair deficient conditions and strong clastogenic potency was not found for any other chemical of the foregoing three groups. Mutagens belonging to this group are vinyl bromide (Vbr) and vinyl carbamate (VCar).
- **5.** Aflatoxin B1 (AFB1) forms a group by itself. Although the predominant lesion is a *N*7-guanine adduct, excision repair doesn't seem to be involved in repair of the adduct. AFB1 is a potent carcinogen and also in Drosophila germ cells it is a very strong mutagen.

Several mutagens belonging to either class 1, 2 or 3 have been studied also in germ cells of mice. Comparisons of Drosophila data with genetic results from specific locus tests in male mice revealed very close similarities; a most unexpected but exciting outcome.

REFERENCES

Abrahamson, S., Würgler, F.E., De Jongh, C. & Meyer, H.U. (1980) How many loci on the X-chromosome of *Drosophila melanogaster* can mutate to recessive lethals? *Environ. Mutagen.* **2**, 447–453.

Aquirrezabalaga, I., Nivard, M.J.M., Comendador, M.A. & Vogel, E.W. (1995a) Hexamethylmelamine is a potent inducer of deletions in male germ cells of *Drosophila melanogaster*. *Carcinogenesis* **16**, 2679–2683.

Aquirrezabalaga, I., Nivard, M.J.M., Comendador, M.A. & Vogel, E.W. (1995b) The cross-linking

agent hexamethylphosphoramide predominantly induces intra-locus and multi-locus deletions in postmeiotic germ cells of Drosophila. *Genetics* **139**, 649–658.

Ballering, L.A.P., Nivard, M.J.M. & Vogel, E.W. (1993) Characterization of the genotoxic action of three structurally related 1,2-dihaloalkanes in *Drosophila melanogaster*. *Mutation Res.* **285**, 209–217.

Ballering, L.A.P., Nivard, M.J.M. & Vogel, E.W. (1994) Mutation spectra of 1,2-dibromoethane, 1,2-dichloroethane and 1-bromo-2-chloroethane in excision repair proficient and repair deficient strains of *Drosophila melanogaster*. *Carcinogenesis* **15**, 869–875.

Ballering, L.A.P., Nivard, M.J.M. & Vogel, E.W. (1996) Characterization by two-endpoint comparisons of the genetic toxicity profiles of vinyl chloride and related etheno-adduct forming carcinogens in Drosophila. *Carcinogenesis* **17**, 1083–1092.

Ballering, L.A.P., Nivard, M.J.M. & Vogel, E.W. (1997) Preferential formation of deletions following *in vivo* exposure of postmeiotic *Drosophila* germ cells to the DNA etheno-adduct forming carcinogen vinyl carbamate. *Environ. Mol. Mutagen.* **30**, 321–329.

Barbin, A. & Bartsch, H. (1986) Mutagenic and promutagenic properties of DNA adducts formed by vinyl chloride metabolites; in *The Role of Cyclic Nucleic Acid Adducts in Carcinogenesis and Mutagenesis* (Singer, B. & Bartsch, H., eds.) pp. 345–358, IARC, Lyon.

Barbin, A. & Bartsch, H. (1989) Nucleophilic selectivity as a determinant of carcinogenic potency (TD_{50}) in rodents: A comparison of mono- and bi-functional alkylating agents and vinyl chloride metabolites. *Mutation Res.* **215**, 95–106.

Bartsch, H., Terracini, B., Malaveille, Ch., Tomatis, L., Wahrendorf, J., Brun, G. & Dodet, B. (1983) Quantitative comparisons of carcinogenicity, mutagenicity and electrophilicity of 10 direct-acting alkylating agents and of the initial O^6:N^7-alkylguanine ratio in DNA with carcinogenic potency in rodents. *Mutation Res.* **110**, 181–219.

Bartsch, H., Barbin, A., Marion, M.J., Nair, J. & Guichard, Y. (1994) Formation, detection, and role in carcinogenesis of etheno bases in DNA. *Drug Metab. Rev.* **26**, 349–371.

Beranek, D.T. (1990) Distribution of methyl and ethyl adducts following alkylation with monofunctional alkylating agents. *Mutation Res.* **231**, 11–30.

Boyd, J.B., Golino, M.D., Nguyen, T.D. & Green, M.M. (1976) Isolation and characterization of X-linked mutants of *Drosophila melanogaster* which are sensitive to mutagens. *Genetics* **84**, 485–506.

Boyd, J.B., Snyder, R.D., Harris, P.V., Presley, J.M., Boyd, S.F. & Smith, P.D. (1982) Identification of a second locus in *Drosophila melanogaster* required for excision repair. *Genetics* **100**, 239–257.

Cizeau, J., Decoville, M., Leng, M. & Locker, D. (1994) Deletions induced in the *white* and *vermilion* genes of *Drosophila melanogaster* by the antitumor drug *cis*-dichlorodiammineplatinum(II). *Mutation Res.* **311**, 31–38.

Dekant, W. & Vamvakas, S. (1993) Glutathione-dependent bioactivation of xenobiotics. *Xenobiotica* **23**, 873–887.

Fedtke, N., Boucheron, J.A., Walker, V.E. & Swenberg, J.A. (1990) Vinyl chloride-induced DNA adducts. II: Formation and persistence of 7-(2"-oxoethyl)guanine and N^2, 3-ethenoguanine in rat tissue DNA. *Carcinogenesis* **11**, 1287–1292.

Friend, S.H., Bernards, R., Rogelj, S., Weinberg, R.A., Rapaport, J.M., Albert, D.M. & Dryja, T.P. (1986) A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma. *Nature* (London) **323**, 643–646.

Gatti, M. (1982) Sister chromatid exchanges in Drosophila; in *Sister Chromatid Exchange* (Wolff, S., ed.) pp. 267–296, John Wiley and Sons, New York.

Graf, U., Vogel, E.W., Biber, U.P. & Würgler, F.E. (1979) Genetic control of mutagen sensitivity in *Drosophila melanogster*. A new allele at the *mei-9* locus on the X-chromosome. *Mutation Res.* **59**, 129–133.

International Agency for Research on Cancer (1976) *IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Man: Some Naturally Occuring Substances;* vol. 10, pp. 51–72, IARC Scientific Publications, Lyon.

Koga, N., Inskeep, P.B., Harris, T.M. & Guengerich, F.P. (1986) *S*-[2-(N^7-Guanyl)ethyl]glutathione, the major DNA adduct formed from the 1,2 dibromoethane. *Biochemistry* **25**, 2192–2198.

Laib, R.J. (1986) The role of cyclic base adducts in vinyl chloride-induced carcinogenesis: Studies on nucleic acid alkylation *in vivo;* in *The Role of Cyclic Nucleic Acid Adducts in Carcinogenesis and Mutagenesis* (Singer, B. & Bartsch, H., eds.) no. 70, pp. 101–108, Scientific Publ. IARC, Lyon.

Lamb, M.J. & Lilly, L.J. (1971) Induction of recessive lethals in *Drosophila melanogaster* by aflatoxin B1. *Mutation Res.* **11**, 430–433.

Lee, W.R. & Kelley, M.R. (1986) Correction for differences in germ cell stage sensitivity in risk assessment; in *Risk Assessment in Relation to Environmental Mutagens and Carcinogens* (Oftedal, P. & Brøgger, A., eds.) pp. 99–102, Alan R. Liss, New York.

Lindsley, D.L. & Tokuyasu, K.T. (1980) Spermatogenesis; in *The Genetics and Biology of Drosophila* (Ashburner, M. & Wright, T.R.F., eds.) vol. 2, pp. 225–294, Academic Press, London.

Malkin, D., Li, F.P., Strong, L.C., Fraumeni, J.F., Nelson, C.E., Kim, D.H., Kassel, J., Gryka, M.A., Bischoff, F.Z., Tainsky, M.A. & Friend, S.H. (1990) Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas and other neoplasms. *Science* **250**, 1233–1238.

Marker, P.C., Seung, K., Bland, A.E., Russell, L.B. & Kingsley, D.M. (1997) Spectrum of *Bmp5* mutations from germline mutagenesis experiments in mice. *Genetics* **145**, 435–443.

Nivard, M.J.M., Pastink, A. & Vogel, E.W. (1992) Molecular analysis of mutations induced in the *vermilion* gene of *Drosophila melanogaster* by methyl methanesulfonate. *Genetics* **131**, 673–682.

Nivard, M.J.M., Pastink, A. & Vogel, E.W. (1993) Impact of DNA nucleotide excision repair on methyl methanesulfonate-induced mutations in *Drosophila melanogaster*. *Carcinogenesis* **14**, 1585–1590.

Nivard, M.J.M., Pastink, A. & Vogel, E.W. (1996) Mutational spectra induced under distinct excision repair conditions by the 3 methylating agents *N*-methyl-*N*-nitrosourea, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and *N*-nitrosodimethylamine in postmeiotic male germ cells of Drosophila. *Mutation Res.* **352**, 97–115.

Nomura, T. (1979) Potent mutagenicity of urethan (ethyl carbamate) gas in *Drosophila melanogaster*. *Cancer Res.* **39**, 4224–4227.

Pastink, A., Vreeken, C., Nivard, M.J.M., Searles, L.L. & Vogel, E.W. (1989) Sequence analysis of *N*-ethyl-*N*-nitrosourea-induced *vermilion* mutations in *Drosophila melanogaster*. *Genetics* **123**, 123–129.

Pastink, A., Heemskerk, E., Nivard, M.J.M., van Vliet, C.J. & Vogel, E.W. (1991) Mutational specificity of ethyl methanesulfonate in excision-repair-proficient and -deficient strains of *Drosophila melanogaster*. *Mol. Gen. Genet.* **229**, 213–218.

Russell, L.B., Cumming, R.B. & Hunsicker, P.R. (1984) Specific-locus mutation rates in the mouse following inhalation of ethylene oxide, and application of the results to estimation of human genetic risk. *Mutation Res.* **129**, 381–388.

Russell, L.B. & Rinchik, E.M. (1993) Structural differences between specific-locus mutations induced by different regimes in mouse spermatogonial stem cells. *Mutation Res.* **288**, 187–195.

Sega, G.A. (1990) Molecular targets, DNA breakage, and DNA repair: Their roles in mutation induction in mammalian germ cells; in *Biology of Mammalian Germ Cell Mutagenesis* (Allen, J.W., Bridges, B.A., Lyon, F.M., Moses, M.J. & Russell, L.B., eds.) Banbury Report 34, pp. 79–91, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Segerbäck, D. (1990) Reaction products in hemoglobin and DNA after *in vitro* treatment with

ethylene oxide and *N*-(2-hydroxyethyl)-*N*-nitrosourea. *Carcinogenesis* **11**, 307–312.

Shukla, P.T. & Auerbach, C. (1980) Genetic tests for the detection of chemically induced small deletions in Drosophila chromosomes. *Mutation Res.* **72**, 231–243.

Sierra, L.M., Pastink, A., Nivard, M.J.M. & Vogel, E.W. (1993) DNA base sequence changes induced by diethyl sulfate in postmeiotic male germ cells of *Drosophila melanogaster*. *Mol. Gen. Genet.* **237**, 370–374.

Smith, P.D., Baumen, C.F. & Dusenbery, R.L. (1983) Mutagen sensitivity of *Drosophila melanogaster*, VI. Evidence from the excision-defective *mei-9*^{AT1} mutant for the timing of DNA-repair activity during spermatogenesis. *Mutation Res.* **108**, 175–184.

Swain, C.G. & Scott, C.B. (1953) Quantitative correlation of relative rates: Comparison of hydroxide ion with nucleophilic reagents towards alkyl halides, esters, epoxides and acylhalides. *J. Am. Chem. Soc.* **75**, 141–147.

Tomatis, L., Narod, S. & Yamasaki, H. (1992) Transgeneration transmission of carcinogenic risk. *Carcinogenesis* **13**, 145–151.

Verburgt, F.G. & Vogel, E. (1977) Vinyl chloride mutagenesis in *Drosophila melanogaster*. *Mutation Res.* **48**, 327–336.

Vogel, E.W. (1989) Nucleophilic selectivity as a determinant of enhanced mutational response in excision repair-defective strains in Drosophila: Effects of 30 carcinogens. *Carcinogenesis* **10**, 2093–2106.

Vogel, E.W., Blijleven, W.G.H., Kortselius, M.J.H. & Zijlstra, J.A. (1982) A search for some common characteristics of the effects of chemical mutagens in Drosophila. *Mutation Res.* **92**, 69–87.

Vogel, E.W., Barbin, A., Nivard, M.J.M. & Bartsch, H. (1990) Nucleophilic selectivity of alkylating agents and their hypermutability in Drosophila as predictors of carcinogenic potency in rodents. *Carcinogenesis* **11**, 2211–2217.

Vogel, E.W., Zijlstra, J.A. & Nivard, M.J.M. (1993) Genetic method for pre-classification of genotoxins into monofunctional or cross-linking agents. *Environ. Mol. Mutagen.* **21**, 319–331.

Vogel, E.W. & Natarajan, A.T. (1995) DNA damage and repair in somatic and germ cells *in vivo*. *Mutation Res.* **330**, 183–208.

Vogel, E.W., Nivard, M.J.M., Ballering, L.A.B., Bartsch, H., Barbin, A., Nair, J., Commendador, M.A., Sierra, L.M., Aquirrezabalaga, I., Tosal, L., Ehrenberg, L., Fuchs, R.P.P., Janel-Bintz, R., Maenhaut-Michel, G., Montesano, R., Hall, J., Kang, H., Miele, M., Thomale, J., Bender, K., Engelbergs, J. & Rajewsky, M.F. (1996) DNA damage and repair in mutagenesis and carcinogenesis: Implications of structure-activity relationships for cross-species extrapolation. *Mutation Res.* **113**, 177–218.

Vogel, E.W. & Nivard, M.J.M. (1997) The response of germ cells to ethylene oxide, propylene oxide propylene imine and methyl methanesulfonate is a matter of cell stage-related DNA repair. *Environ. Mol. Mutagen.* **29**, 124–135.

Wogan, G.N. (1973) in *Methods in Cancer Research*, (Busched. H., ed.) vol. 7, pp. 309–344, Academic, New York.

Zijlstra, J.A. & Vogel, E.W. (1988) The ratio of induced recessive lethals to ring-X loss has prognostic value in terms of functionality of chemical mutagens in *Drosophila melanogaster*. *Mutation Res.* **201**, 27–38.