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Genotoxicity of Glycol Ethers

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The genetic toxicology of glycol ethers is reviewed. Ethylene glycol monomethyl ether (EGME) and diglyme have been more extensively studied than other members of this series. Most results indicate a lack of genotoxic potential, but certain tests have yielded positive responses with certain compounds. Ethylene glycol monoethyl ether (EGEE) induced sister chromatid exchanges and chromosomal aberrations in cultured cells. Both EGME and diglyme induced mouse sperm head morphological changes, male rat weak dominant lethal mutations and marked, but reversible, loss of male rat fertility.

Introduction

The glycol ethers are water-soluble compounds with widespread usage in industries, e.g., chemical, food, printing, publishing, paper, electronics, mining and furniture. They are, basically, aliphatic compounds with at least one ether group. They also frequently contain hydroxyl groups, although these may be esterified with carboxylic or mineral acids. Commercial samples always contain small quantities of peroxides, which form at the ether group, as readily demonstrated by the oxidation of potassium iodide solutions. The general toxicology of this class of chemicals has been reviewed (*1*), but it is only recently that results of mutagenic studies conducted in a number of laboratories have become available. The purpose of this paper is to review this work and provide some additional information derived from *in vitro* tests (Table 1).

Results

Bacteria

There is no evidence of mutagenic activity of several glycol ethers in the Ames test using various commonly used strains of *S. typhimurium*. Ethylene glycol monomethyl ether (EGME, CAS No. 109-86-4) was not active in either the standard plate assay or the preincubation modification at dose levels up to 33 mg per plate (plate volume 20 mL), with or without a homogenized rat liver 9000*g* supernatant fluid activation system (rat S9 mix). The strains used were TA1535, TA100, TA1537, TA1538 and TA98 (*2*). The assay has been rerun recently in the same laboratory using *S. typhimurium* TA102, which can be reverted by a number of oxidizing agents, including several peroxides. EGME was not mutagenic in this strain (McGregor and Prentice, unpublished data, 1983).

Ethylene glycol ethyl ether (EGEE, CAS No. 110-80-5) was not mutagenic in *S. typhimurium* TA1538 (*3*), or *E. coli* scl-4-73 (*4*). It was also tested in a preincubation assay with *S. typhimurium* TA1535, TA100, TA1537, TA1538 and TA98 as part of a National Toxicology Program (NTP) study (S. Haworth, personal communication). No toxic or mutagenic response was obtained at dose levels up to 10 mg per plate in either the absence or presence of rat S9 mix or hamster S9 mix. Testing of other glycol ethers has been more limited. Propylene glycol monomethyl ether (PGME, CAS No. 107-98-2) was not mutagenic in the Ames test (Dow Chemical Co., unpublished data, 1983), and the EGME condensation product, bis(2-methoxyethyl)ether (diglyme CAS No 111-96-6), was not mutagenic in *S. typhimurium* strains TA1535, TA100, TA1537, TA1538 and TA98 at dose levels up to 94 mg per plate (McGregor et al., unpublished data, 1983). Ethylene glycol dimethyl ether (EGDiME, CAS No. 110-71-4) has not been subjected to mutagenicity tests, but it has a cytotoxicity profile towards *S. typhimurium* TA100 similar to acetone, in that it causes no toxicity at 200 μL per plate, but kills the culture at 500 μL per plate (*7*).

A limitation of the activation system used in Ames' test is the emphasis placed upon the microsomal mixed-function oxidase type of reaction and the absence of opportunity for alcohol oxidation by dehydrogenase enzymes. In an effort to overcome this limitation, experiments were performed in which *S. typhimurium* strains were exposed to EGME in the presence of equivalent quantities of β-nicotinamide-adenine dinucleotide (βNAD^+) and 150 units alcohol dehydrogenase per plate. Initial experiments indicated that EGME metabolites, or βNAD^+ was toxic to the bacteria at dose levels in excess of about 125 μg EGME and 1.12 mg

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Table 1. Results of mutagenicity tests with glycol ether.

Test type	Compound	Test	Result[a]	Reference
Bacterial mutation	EGME	*S. typhimurium* TA1535, TA100, TA1537, TA1538, TA98 with and w/o S9 mix and with alcohol dehydrogenase	–	(*2*)
	EGME	*S. typhimurium* TA102 with and w/o S9 mix and with alcohol dehydrogenase	–	McGregor and Prentice (unpublished) and this paper
	EGEE	*S. typhimurium* TA1535, TA100, TA1537, TA1538, TA98, without S9 mix and with rat S9 mix and hamster S9 mix	–	NTP (unpublished); S. Haworth, personal communication
	EGEE	*S. typhimurium* TA1538	–	(*3*)
	EGEE	*E. coli* scl-4-73	–	(*4*)
	PGME	*S. typhimurium* TA1535, TA100, TA1537, TA1538, TA98, with and w/o rat S9 mix	–	Dow Chemical Co. (unpublished)
	Diglyme	*S. typhimurium* TA1535, TA100, TA1537, TA1538, TA98, with and w/o rat S9 mix	–	(*2*)
	Methoxyacetic acid	*S. typhimurium* TA1535, TA100, TA1537, TA1538, TA98, no activation	–	(*2*)
Yeast, mutation	EGME	*Schizosaccharomyces pombe*	–	(*5*)
Mammalian, *in vitro*, unscheduled DNA synthesis	EGME	Fibroblasts, grain counts, with and w/o rat S9 mix	–	(*2*)
	EGBE	Primary hepatocyte, scintillation counting	?	(*6*)
	PGME	Primary hepatocyte, grain counts	–	Dow Chemical Co. (unpublished)
	Diglyme	Fibroblasts, grain counts, with and w/o rat S9 mix	–	(*2*)
Mammalian, *in vitro*, sister chromatic exchange	EGEE	CHO cells with and w/o rat S9 mix	+ (weaker with rat S9 mix)	NTP (unpublished); S. Galloway, personal communication
	EGBE	CHO cells with and w/o rat S9 mix	–	(*6*)
Mammalian, *in vitro*, chromosomal aberrations	EGEE	CHO cells with and w/o rat S9 mix	+ (weaker with rat S9 mix)	NTP (unpublished); S. Galloway, personal communication
	PGME	CHO cells	–	Dow Chemical Co. (unpublished)
Mammalian, *in vitro*, point mutations	EGME	L5178Y mouse lymphoma $TK^{+/-}$cells with rat S9 mix	–	This paper
	EGBE	CHO cells, HGPRT locus, with and w/o rat S9 mix	–	(*6*)
Drosophila, sex-linked recessive lethal	EGME	3-day-old male OrK stocks, dynamic atmosphere of 25 ppm for 1 hr or 500 ppm for 15 min	?	(*2*)
	EGEE	3 day-old-males, feeding and injection	–	NTP (unpublished)
	Diglyme	3-day-old male OrK stocks, dynamic atmosphere of 250 ppm for 2.75 hr	?	(*2*)
Rat bone marrow cytogenetics	EGME	Dynamic atmospheres of 25 or 500 ppm, 7 hr/day, for either 5 days with sampling 6 hr later or 1 day with sampling 6 hr, 24 hr or 48 hr later	–	(*2*)
	Diglyme	Dynamic atmospheres of 250 or 1000 ppm, 7 hr/day for either 5 days with sampling 6 hr later or 1 day with sampling 6 hr, 24 hr or 48 hr later	–	(*2*)
	Isobutyl ether mixture of propylene glycols (Dowanol PIB-T)	Dynamic atmospheres of 200 ppm, 7 hr/day for 4 weeks	–	Dow Chemical Co. (*1*)
Mouse sperm abnormality test	EGME	Dynamic atmospheres of 25 or 500 ppm, 7 hr/day for 5 days, with sampling 35 days later	+	(*2*)
	Diglyme	Dynamic atmospheres of 250 ppm for 7 hr/day for 5 days, or 1000 ppm for 7 hr/day for 4 days, with sampling 35 days later	+	(*2*)
Male rat dominant lethal test	EGME	Dynamic atmospheres of 30, 100 or 300 ppm for 6 hr/day, 5 days/week for 13 weeks	Male sterility at 300 ppm reversible	(*9*)
	EGME	Dynamic atmospheres of 25 or 500 ppm for 7 hr/day for 5 days, followed by 10 successive weekly matings	± High dose, male sterility at week 5, reversible	(*2*)
	Diglyme	Dynamic atmospheres of 250 or 1000 ppm for 7 hr/day for 5 days, followed by 10 successive weekly matings	± High dose, low male fertility at weeks 5–7, reversible	(*2*)

[a]Results: – no significant response; + significant response; ± significant response, but weak; ? unclear or not reproducible, further testing needed.

βNAD$^+$ per plate (*2*). Later tests, in which *S. typhimurium* TA102 as well as TA98 and TA100 were used, tended to support these earlier results (Table 2). However, the level of βNAD$^+$ was restricted to 425 μg per plate, at which level some toxicity was observed. This restriction imposed a limit on oxidizable EGME of 49 μg per plate.

Yeast

EGME was examined for its potential to induce forward mutations in the fission yeast, *Schizosaccharomyces pombe* (*5*). While there was some weak cytotoxicity observed following exposure in the medium at 10% (v/v), no mutation induction occurred.

Mammalian Cells

Unscheduled DNA Synthesis (UDS). UDS assays have been performed with EGME, diglyme, PGME and ethylene glycol monobutyl ether (EGBE). EGME did not induce UDS in human embryo fibroblasts in either the presence or absence of Aroclor-induced male rat liver at dose levels up to 10 mg/mL. Diglyme also failed to induce observable effects in the same test system at doses up to 19 mg/mL (*2*). Using a rat primary hepatocyte UDS assay, PGME did not induce any increases in grain counts (Dow Chemical Co.). Ethylene glycol monobutyl ether (EGBE, CAS No. 111-76-2) has also been tested in a rat primary hepatocyte test, although total radioactivity was measured by scintillation counting, rather than by nuclear grain counts. EGBE was tested at concentrations up to 0.1% of the culture medium for 2 hr. Statistically significant increases occurred in nuclear and DNA-associated radioactivity at two low dose levels, the maximum response being about 1.3 times the control levels. The investigations suggested that EGBE might be inhibiting UDS at higher concentrations (*6*). While this possibility should be conceded, it would appear advisable to attempt a confirmation of this response.

Table 2. Ames' *Salmonella*/activation assay of ethylene glycol monomethyl ether, using *S. typhimurium* TA98, TA100 and TA102.[a]

Compound per plate, μg	Average mutant counts ± SD (triplicate plates)[b]		
	TA98	TA100	TA102
0	24 ± 4	75 ± 4	97 ± 15
100	28 ± 10	71 ± 4	101 ± 11
333	31 ± 2	74 ± 13	97 ± 11
1,000	23 ± 1	70 ± 14	104 ± 11
3,333	30 ± 11	62 ± 4	128 ± 11
10,000	28 ± 8	71 ± 14	122 ± 17
33,333	20 ± 3	63 ± 2	122 ± 7
100,000	24 ± 6	70 ± 1	123 ± 1

[a]A pre-incubation (5 min, 37°C) mixture consisted of: 0.1 mL bacteria, 150 units alcohol dehydrogenase, 425 μg βNAD$^+$ and 100 μL test solution. After the addition of 2 mL soft agar, the mixture was poured onto Vogel-Bonner plates and incubated for 3 days at 37°C.

[b]The quantity of βNAD$^+$ used was slightly toxic to the cells.

Sister Chromatid Exchange (SCE). EGEE was tested in an NTP study at concentrations up to 9 mg/mL in which it did induce SCE in Chinese hamster ovary (CHO) cells in both the presence and absence of rat S9 mix. The response was weaker in the presence of rat S9 mix than in its absence (NTP, unpublished results; S. Galloway, personal communication). EGBE did not induce SCE in CHO cells exposed to concentrations up to 0.25% for 2 hr in the presence and 5 hr in the absence of S9. These experiments were performed twice (*6*).

***Chromosomal Aberration Induction* in Vitro.** EGEE induced chromosomal aberrations in CHO cells in the absence of rat S9 mix, but failed to do so in its presence (NTP, unpublished results; S. Galloway, personal communication). This result is similar to that obtained in the SCE test, in that the presence of rat S9 mix reduced the response. PGME is not clastogenic toward

Table 3. L5178Y TK$^{+/-}$ Mouse lymphoma mutagenesis assay of ethylene glycol monomethyl ether.[a]

Compound concentration, μg/mL	Cell concentration (× 10^5)		Suspension growth		Cloning growth				
	Day 1	Day 2	Total	% Control	Average viable count	% Control	% Total growth[b]	Average mutant count	Mutant frequency × (10^{-5})
0	7.7	11.8	10.1	100	136	100	100	24	3.2
0.01	6.6	11.8	8.7	86	157	115	99	26	3.3
0.03	9.2	8.3	8.5	84	160	123	103	24	3.0
0.10	5.7	11.2	7.1	70	176	118	83	31	3.5
0.33	8.7	8.5	8.2	81	153	113	92	34	4.4
1.00	5.7	13.6	8.6	85	150	110	94	37	4.9
3.33	5.6	7.9	4.9	49	239	176	86	34	2.8
10.00	7.5	9.3	7.8	77	170	125	96	31	3.6
33.33	9.3	9.9	10.2	101	132	97	98	19	2.9
100.00	7.5	10.8	9.0	89	158	116	103	32	4.1
2.5[c]	4.7	8.5	4.4	44	61	45	20	32	10.5

[a]Exposures were for 4 hr in the presence of Aroclor 1254-induced male rat liver S9 mix (10% v/v incubation mixture). The expression period was 2 days. Selection was with 3 μg/mL trifluorothymidine.

[b](Relative suspension growth × relative cloning efficiency) ÷ 100.

[c]Cyclophosphamide.

CHO cells, although experimental details are not available (Dow Chemical Co., unpublished data, 1983).

***Point Mutation Induction* in Vitro.** EGME has been tested on a single occasion in the L5178Y mouse lymphoma TK+/– cell assay in the presence of rat S9 mix (Table 3). At doses up to 10 mg/mL for 4 hr there was no mutation induction. EGBE was not mutagenic at the HGPRT locus of CHO cells at concentrations up to 1% of the culture medium for 5 hr, followed by a 7-day expression period (*6*). The experiment was performed in both the presence and absence of S9. A single, high mutation frequency occurred in the presence of S9 at the lowest concentration used, but there was no suggestion of a dose-related response.

Drosophila

Sex-linked recessive lethal (SLRL) test results have been reported for EGME and diglyme (*2*), although experiments with EGEE have been completed in an NTP study. Two independent stocks of flies were exposed to EGME or diglyme vapors in dynamic atmospheres. EGME exposure conditions were 25 ppm for 1 hr or 500 ppm for 15 min, while diglyme was tested at a single concentration, 250 ppm, for 2.75 hr. In the experiment with EGME, mutation frequencies were relatively high, but inconsistencies in the results obtained with the two fly stocks suggested that EGME might not be responsible. Several small, follow-up studies were performed, but these failed to clarify the position. It was suggested that the metabolic status of the flies (particularly with regard to alcohol metabolism) was contributing to the lack of reproducibility (*8*). Similar problems arose with diglyme, and the authors concluded that this compound should be reinvestigated in the SLRL test. EGEE was nonmutagenic in the NTP study.

Cytogenetic Analysis of Rat Bone Marrow Cells

EGME, diglyme and an isobutyl ether mixture of propylene glycols have been subjected to this type of test. In the tests with EGME and diglyme, groups of 10 male and 10 female rats were exposed to the test compound vapors 7 hr/day for either 1 or 5 days (*2*). Vapor concentrations used were 25 or 500 ppm EGME and 250 or 1000 ppm diglyme. The 1-day exposed groups were killed at 6, 24 or 48 hr after the end of exposure, while the 5-day exposed rats were killed 6 hr after the end of exposure. Significant increases in aberrant cell frequency occurred in female rats exposed to 25 ppm EGME ($p < 0.01$) and male rats exposed to 250 ppm diglyme ($p < 0.05$). The restriction to a single sex and, more important, the lack of response at the higher concentration of either compound led the authors to conclude that the elevations were not induced by the test compound. Dowanol PIB-T, which is a mixture of the isobutyl ethers of propylene glycol (70%), dipropylene glycol (20–25%) and tripropylene glycol (5–10%), was tested for clastogenic potential in rats (*1*). Rats were exposed to 200 ppm vapors 7 hr/day for 4 weeks, after which bone marrow metaphase chromosomes were prepared and examined. No significant effects were observed and, while the experiment was inconclusive, the data suggested an absence of any clastogenic potential.

Mouse Sperm Abnormality Test

EGME and diglyme have been tested in this system (*2*). Groups of 10 $B6C3F_1$ male mice were exposed 7 hr/day to 25 ppm EGME, 500 ppm EGME or 250 ppm diglyme for 5 days or 1000 ppm diglyme for 4 days (exposure being shortened in this last group because of toxicity). Five weeks later epididymal sperm were collected and examined for head morphological abnormalities. Significant increases in abnormalities were observed in the 500 ppm EGME and the 1000 ppm diglyme groups. The greatest increases were in the amorphous head grouping, which increased from 2.02% to 5.11% following 500 ppm EGME exposure and from 2.18% to 20.87% following 1000 ppm diglyme exposure. No significant effects were observed at the lower vapor concentration levels of either compound.

Dominant Lethal Test

EGME and diglyme have been submitted to male rat (CD strain) dominant lethal tests. In one test with EGME, rats were exposed to 30, 100 or 300 ppm EGME 6 hr/day, 5 days/week for 13 weeks, then mated with unexposed females (*9*). Fertility was totally suppressed in the 300 ppm group immediately after exposure, while the fertility of males exposed to 30 or 100 ppm was indistinguishable from the controls. Furthermore, fertility of the 300 ppm exposed males was fully restored 13 weeks after the end of exposure.

There was no evidence for a dominant lethal effect in the 30 or 100 ppm groups immediately after exposure or in the 300 ppm group 13 or 19 weeks after exposure.

EGME and diglyme were also studied for their dominant lethal inducing potential following the exposure conditions described above for the bone marrow clastogenic studies (*2*). No adverse effects were observed in male rats exposed to either 25 ppm EGME or 250 ppm diglyme. In the 500 ppm EGME group mating weeks 1 and 2 were normal with regard to pregnancy and implantation frequencies, but reproductive performance became progressively poorer with successive weekly matings until in week 6 there were no pregnancies (Fig. 1). Recovery began in week 7 and normal activity was regained by week 9. The effects of 1000 ppm diglyme were similar but not identical (Fig. 2). Pregnancy frequency was not consistently affected up to mating week 3, which was followed in weeks 5, 6 and 7 by pregnancy frequencies of about 10% and full

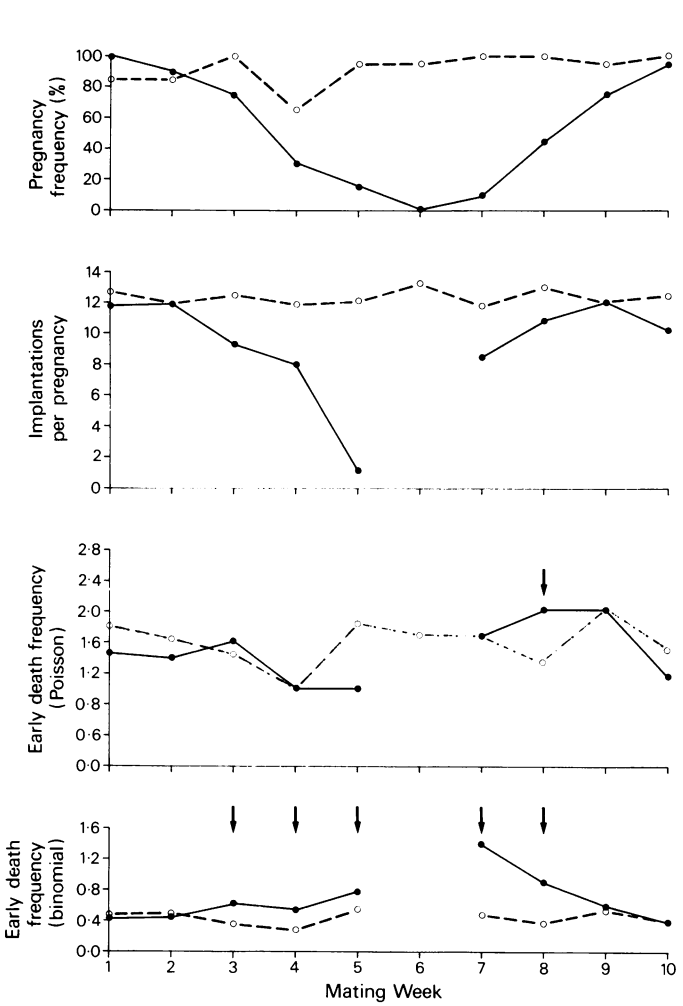

FIGURE 1. Male rat dominant lethal mutation assay with ethylene glycol monomethyl ether: (○) air control. (●) 500 ppm, 7 hr/day, 5 days. (↓) $p < 0.05$.

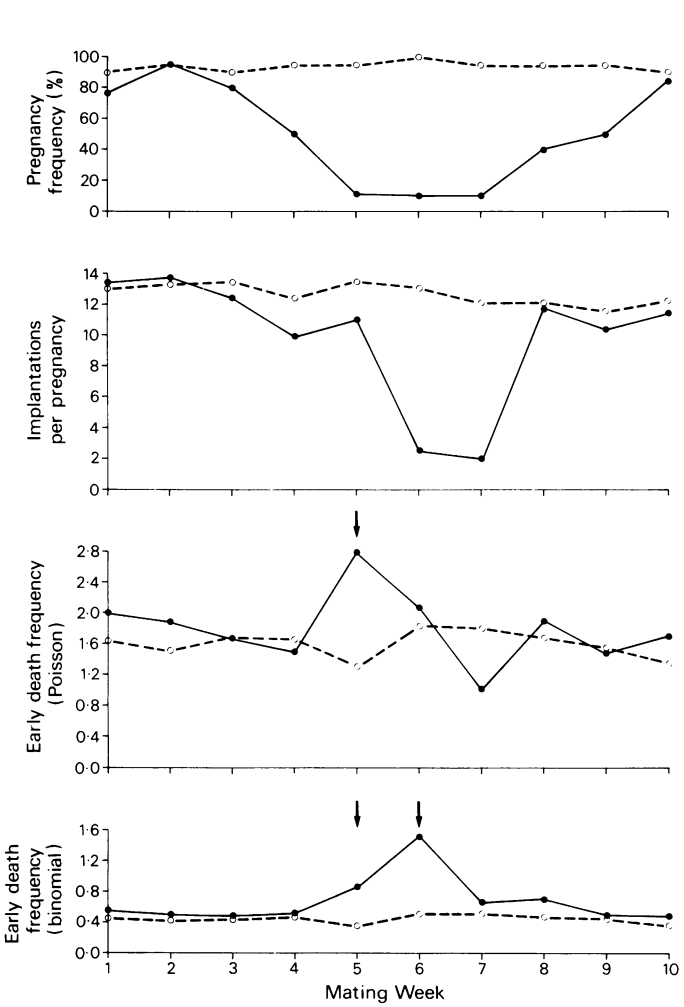

FIGURE 2. Male rat dominant lethal mutation assay with diglyme: (○) air control; (●) 1000 ppm, 7 hr/day, 5 days. (↓) $p < 0.05$.

recovery in week 10. Implantations were notably reduced only in mating weeks 6 and 7.

In these experiments it was difficult to conclude whether or not there had been any dominant lethal effects. Post-implantation early deaths were increased in the 500 ppm EGME group only in week 8, if the Freeman-Tukey Poisson transformation model was adopted, whereas significant increases were recorded in weeks 3, 4, 5, 7 and 8 when the Freeman-Tukey binomial transformation model was used. Early death increases were significant also in the 1000 ppm diglyme group in week 5 using the Poisson transformation and weeks 5 and 6 when using the binomial transformation. While there are reasons why these distribution models lead to different conclusions, perhaps most weight should be placed upon results which are significant irrespective of the model used. Furthermore, implantation numbers were low in several of the mating weeks where significant increases in the proportion of early deaths were recorded. Studies in CD-1 mice have shown that, in untreated animals, there are proportionally more early deaths in females with low numbers of implantations (*10*). The same is true of CD rats (Fig. 3). In data pooled from 16 experiments, the mean percentage early deaths in untreated or vehicle control rats fell from 50% in pregnancies with a single implantation to about 7% when there were more than five implantations. Consequently, the high proportion of early deaths in week 5 of the 500 ppm EGME group and week 6 of the 1000 ppm diglyme group could be at least partly explained in terms of the low implantation frequency. Nevertheless, there were residual dominant lethal effects which could not be explained in this way or minimized because of the model dependency for their statistical significance. A reservation which might be expressed about the origins of these effects is that the clearest EGME response was observed in week 8, while the clear diglyme response was restricted to week

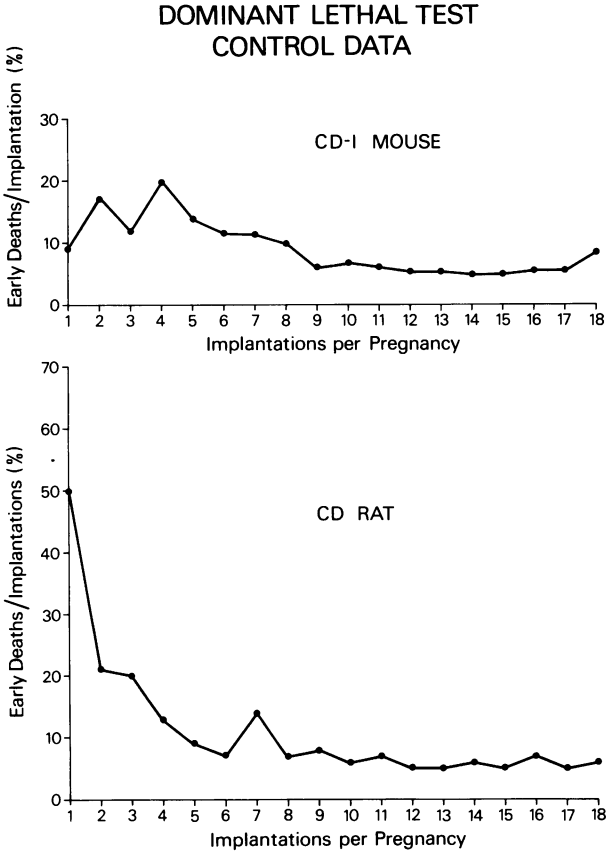

FIGURE 3. Pooled dominant lethal mutation assay control data indicating the relationship between the proportion of early deaths and implantation numbers per pregnancy.

5. Such a reservation can only be based on an assumption that any effects of these compounds should have a similar pathogenesis.

Discussion

Metabolic studies upon glycol ethers now suggest that the primary alcohols are largely oxidized to the corresponding aldehydes and acids, while secondary alcohols may be preferentially dealkylated to the corresponding glycols. Thus, EGME is oxidized to methoxyacetic acid, while PGME is demethylated to propylene glycol (*11*). EGBE is oxidized to *n*-butoxyacetic acid in several species, including man (*12*), and EGisoPE is oxidized to isopropoxyacetic acid (*13*). While both types of reaction may occur *in vivo*, the co-factor (βNAD^+) required for alcohol metabolism is not usually supplied to *in vitro* test systems. Consequently, it is possible that the primary alcohols have been inadequately tested *in vitro*. A difficulty in using an alcohol dehydrogenase-based activation system is that βNAD^+ is toxic to cells and, in the very few experiments where this activation system has been supplied, no attempt was made to regenerate the co-factor. The oxidation of the primary alcohol was limited, therefore, by the concentration of βNAD^+. Until a suitable βNAD^+ regenerating system is devised, this unsatisfactory position will persist. The lack of any mutagenic response in the Ames test with methoxyacetic acid is reassuring to some extent, but methoxyacetaldehyde is likely to be a more reactive compound.

In vitro test results have generally indicated a lack of genotoxic potential in this class of chemicals, but certain equivocal or positive responses have been obtained (Table 1). Equivocal results were obtained with EGBE in the SCE test with S9 and the rat hepatocyte UDS assay. Positive results were obtained with EGEE in both the SCE test and CHO cell chromosomal aberration test in the absence of S9. Since so few studies have been performed, placing these results in a chemical class context is difficult and would be premature. It is quite possible that the clastogenic potential is confined to EGEE. This should be tested in a program which sets out specifically to compare a series of glycol ethers in clastogenic and SCE tests within a laboratory.

While Drosophila SLRL experiments with EGEE within the National Toxicology Program did not show any increases in mutation frequency, the reported tests with EGME and diglyme suffered from lack of reproducibility, and the authors recommended that those studies should be extended before any conclusions were drawn.

However, clearer indications of an impaired function has come from dominant lethal tests and sperm abnormality tests with EGME and diglyme. The reduction in male rat fertility and the occurrence of sperm with abnormal head morphology in mice are beyond dispute. There were also indications of weak dominant lethal mutation induction in mating week 8 following EGME exposure and in mating week 5 following diglyme exposure. Testicular injury caused by EGME and EGEE has been demonstrated in a number of studies (*14–18*).

According to Foster et al. (*18*), cell stages affected by EGME are pachytene through to dividing spermatocytes, but sparing cells in preleptotene, leptotene and zygotene. EGEE and the corresponding methoxyacetic and ethoxyacetic acids have similar effects. It has been suggested that these acids are, in fact, the active metabolites (*11,18*) and that the injury observed is a result of zinc loss from testis, which occurs 4 days after treatment with EGME or methoxyacetic acid (*18*). Zinc concentration has been shown to have an important role in testicular injury induced by cadmium (*19*), and phthalate esters (*20–22*).

An important ancillary observation which supports the hypothesis that the acid metabolites are responsible for testicular damage is that PGME, which is largely *O*-demethylated, has a much lower toxicity. The position of diglyme is potentially anomalous, since it is not an alcohol, yet induces effects similar to those induced by EGME in the dominant lethal and sperm abnormality tests. Possible explanations: (1) the acid metabolites are not responsible for glycol ether toxicity; (2) the

mechanism of diglyme-induced toxic response is different; or (3) diglyme is demethylated and the rsulting primary alcohol is oxidized via the alcohol and aldehyde dehydrogenase enzymes.

The evidence available is sufficient to conclude that the glycol ethers are not strongly genotoxic agents. The effective concentrations of EGEE in the *in vitro* SCE and chromosomal aberrations tests were 6.8 and 9.5 mg/mL, which are considered to be high. *In vivo* effects in the dominant lethal and sperm abnormality tests were observed with 500 ppm, but not 25 ppm EGME, and 1000 ppm, but not 250 ppm diglyme. At these effective doses, other more readily observable types of toxic change occur. Furthermore, as the involvement of a genetic mechanism has not been clearly established, the difficult question of whether or not there is a threshold effect does not arise.

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