Is ethanol genotoxic? A review of the published data

Barry J. Phillips and Peter Jenkinson

A great many studies have been carried out on the toxicology of ethanol, the majority in the context of the effects of the consumption of alcohol in beverages. Published information relevant to the assessment of the possible genotoxic potential of ethanol has been reviewed and evaluated in terms of the safety of ethanol as an industrial chemical, rather than as a component of beverages. The available data on ethanol from standard genotoxicity test methods are incomplete. There is clear evidence that ethanol is not a bacterial or mammalian cell mutagen but in vitro assays for chromosome aberration, although mostly negative, have generally not included exogenous metabolic activation. Evidence from the use of ethanol as a vehicle control suggests that it is not mutagenic or clastogenic in vitro. Reported tests for chromosome aberration induction in vivo are all negative and only a minority of micronucleus tests are positive. Conflicting results have been reported for the dominant lethal assay, although an inter-laboratory study performed to OECD guidelines was negative. There is some evidence that ethanol induces SCE in vivo and can also act as an aneugen at high doses. Many in vivo studies were designed to model alcoholism and used very high doses, sometimes for long periods. Outcomes may have been affected by disturbances of metabolism giving rise to secondary effects. It is concluded that there is no significant evidence that ethanol is a genotoxic hazard according to the criteria normally applied for the purpose of classification and labelling of industrial chemicals. Some degree of genotoxicity may result from excessive alcohol drinking, but this is not considered relevant to any conceivable exposure obtainable by either inhalation or dermal exposure in the workplace.

Introduction

This review is an assessment and interpretation of the available information on the genotoxicity of ethanol and the context is the use of ethanol as an industrial chemical rather than as a component of alcoholic beverages. It attempts to determine whether or not ethanol exposure in the workplace should be regarded as representing a genotoxic hazard, giving due regard to currently accepted practice in test selection and design for the testing of industrial chemicals. In the first instance, data from the main test systems which are currently used to assess mutagenicity for regulatory purposes is evaluated to determine whether these data alone would indicate that ethanol is a genotoxic hazard. This will enable a comparison to be made with data for other industrial chemicals. For the sake of completeness, supplementary information on the genotoxicity of ethanol in non-standard tests is summarized in an attempt to clarify the nature of its effects, if any, on the genetic apparatus.

In industry ethanol is used very extensively in the manufacture of many products, including plastics, pharmaceuticals and resins, and as a solvent, fuel or intermediate in chemical synthesis. Occupational exposure to ethanol normally occurs by inhalation or skin contact. However, unlike most industrial chemicals, ethanol is consumed by a high proportion of the population of many Western countries, as a constituent of alcoholic beverages. The adverse health effects of excessive alcohol consumption are well known and research on this subject has produced a large body of literature describing the effects of ethanol exposure on humans, animals and in vitro systems (see for example UK Department of Health, 1995). Many of the published experimental studies have investigated the effects of high dose, chronic ethanol exposure with the intention of assessing the consequences of alcoholism. This causes difficulties when attempting to assess the potential mutagenicity of ethanol. Its low acute toxicity (and for in vitro studies its high solubility) has allowed the use of doses that are well above those recommended as maximum dose levels in current testing guidelines. Very high doses of chemicals, particularly when administered chronically, can give rise to secondary effects resulting from generalized toxicity and metabolic disturbance. In cell culture systems physicochemical effects such as osmotic pressure changes are known to induce genetic damage which is of no relevance to the likely effects of the test chemical in vivo (Scott et al., 1991). In relatively few cases have studies relating to the mutagenicity of ethanol been carried out using protocols which would be regarded now as being appropriate and acceptable for testing the mutagenic potential of an industrial chemical. In spite of this, a proposal (European Chemicals Bureau document no. ECBI/74/95-Add 3) was recently considered for the classification and labelling of ethanol as a mutagen under the Dangerous Substances Directive (67/548/EEC) and ethanol has recently been classified by the MAK Commission as a category 2 mutagen.

The possible mutagenicity of ethanol for humans was reviewed in 1987 by an expert group of the International Commission for the Protection against Environmental Mutagens and Carcinogens (ICPEMC). The report of the group (ICPEMC, 1987) includes a summary of the genotoxicity data available at the time, collated by Obe and Anderson (1987). The data were not considered sufficient to enable a definite conclusion to be drawn concerning the ability of alcohol to

1To whom correspondence should be addressed. Tel: +44 01332 792 896; Fax: +44 01332 799 018; Email: pjenkinson@safepharm.co.uk

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induce heritable mutations in human germ cells and, although the existence of positive genotoxicity test results was noted, the group did not draw conclusions about the possible effects of ethanol in relation to genetic damage in somatic cells.

The International Agency for Research on Cancer (IARC, 1988) found that there was sufficient evidence for the carcinogenicity of alcoholic beverages in humans. It should be emphasized that the IARC report referred to alcoholic beverages and not ethanol per se. Data on the genotoxicity of ethanol were extensively reviewed in this assessment but it is not clear whether or not this information was considered to provide evidence that ethanol, acting through a genotoxic mechanism, was responsible for the carcinogenicity of alcoholic beverages.

In 1995 the UK Department of Health considered the health implications of alcohol consumption (UK Department of Health, 1995). The UK Department of Health Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment reviewed the evidence for the mutagenicity of ethanol, acetaldehyde and alcoholic beverages and concluded that the consumption of alcoholic beverages does not present any significant concern with respect to their mutagenic potential. The overall conclusion of the UK Department of Health was that, for adults, the daily consumption of 8 g ethanol was likely to be beneficial and that, with certain caveats, 24–32 (for men) or 16–24 g/day (for women) would not accrue a significant health risk.

**Results of ‘standard’ genotoxicity tests**

**Bacterial mutation assays**

The data from bacterial mutagenicity assays, particularly the Ames test, are extensive and a number of studies can be regarded as meeting the requirements of current test guidelines. The results have generally been negative (McCann et al., 1975; Cotruvo et al., 1977; Arimoto et al., 1982; Blevins and Taylor, 1982; Blevins and Shelton, 1983; Hayes, 1984; Hellmer and Bolefsoldi, 1992). De Flora et al. (1984a) found a very weak positive effect of ethanol in a DNA repair test with Escherichia coli but no effect in the Ames test with strains TA1535, 1537, 1538, 97, 98 and 100. There was a weak but reproducible positive effect in TA102 (less than 2-fold increase in revertants) but only at ethanol concentrations of 160 and 240 mg/plate (De Flora et al., 1984b). These concentrations are far in excess of the generally accepted maximum concentration for routine testing (5 mg/plate; OECD, 1997, Guideline no. 471). Using a preincubation assay, Zeiger et al. (1992) found negative results at concentrations up to 10 mg/plate in strains TA97, 98, 100, 104 and 1535 with or without Aroclor-induced S9 mix, from both rats and Syrian hamsters, at two concentrations.

It should be borne in mind that many laboratories, including our own, have used ethanol as a vehicle for some test compounds. Historical data from Safepharm Laboratories show no evidence of mutagenicity, with or without S9, for solvent controls with a typical ethanol concentration of 100 μl (79 mg)/plate (see Appendix, Tables A1 and AII).

It can be concluded with some confidence that ethanol is not mutagenic to bacteria.

**Chromosome aberration tests in vitro**

Most of the reported chromosome aberration tests with ethanol have been negative. No chromosome aberrations were found in human lymphocyte cultures (Obe et al., 1977; Konigstein et al., 1984; Banduhn and Obe, 1985) or in human lymphoid cell lines at ethanol concentrations of 8 and 16 mg/ml (174 and 348 mM) (Hsu et al., 1991; Brown et al., 1992). Ethanol administered daily at 0.8 mg/ml (17.4 mM) for 9 days failed to induce chromosome aberrations or micronuclei in HeLa cells (Obe and Ristow, 1979). Negative results have also been reported with Chinese hamster cells (Darroudi and Natarajan, 1987; Lin et al., 1989), although Darroudi and Natarajan found chromosome aberrations induced by ethanol only in the presence of a 2000 g extract of the leaves of Zea mays (maize) with an NADPH-generating cofactor mix. This metabolizing system itself induced chromosome aberrations in Chinese hamster ovary (CHO) cells but also increased the number of aberrations induced by 160 mM ethanol. Tests for micronucleus induction in vitro have been negative (Obe and Ristow, 1979; Lasne et al., 1984). A high level of chromosome aberrations was observed in pre-implantation mouse embryos exposed in vitro to concentrations of ethanol ranging from 1 to 30 mg/ml, equivalent to 22–650 mM (Lau et al., 1991). These concentrations are extremely high and it is possible that chromosome damage resulted from non-specific effects such as high osmotic pressure.

In a study in human lymphocytes, Badr et al. (1977) reported a dose-related increase in chromosome aberrations with ethanol concentrations of 1.16, 2.32 and 3.48 mg/ml (25, 50 and 75 mM) in the absence of metabolic activation. This result is in conflict with most of the other published studies, as are strongly positive results for a mouse dominant lethal assay and a mouse micronucleus test which were reported in the same publication. One possible explanation for these exceptional findings might be contamination of the ethanol with a mutagen. In the case of chromosome aberrations, misclassification is also a possibility as the photographs of aberrations provided in the paper suggest that the identification of some classes of aberration may be questioned. In an abstract Au and Badr (1979) reported that ethanol did not induce chromosome aberrations in either human lymphocytes or CHO cells treated for 12–48 h with 0.5–10 mg ethanol/ml (10.9–217 mM) but that ethanol in the presence of S9 caused a dose-related increase in aberrations in CHO cells.

As with the Ames test, ethanol is sometimes used as a vehicle in chromosome aberration tests. At Safepharm Laboratories solvent controls, commonly corresponding to a concentration of 8 mg/ml (174 mM) ethanol, have consistently demonstrated a lack of chromosome aberration induction in human lymphocyte, CHO and CHL cultures, both with and without metabolic activation (see Appendix, Tables AIV and AV). This concentration is well in excess of that reported to give positive results by Badr et al. (1977).

Most of the studies referred to above are incomplete in design, as currently recommended for screening purposes (OECD, 1997). In particular, they generally did not employ metabolic activation and in most cases did not use a sufficiently wide dose range. There is little evidence, however, that ethanol is clastogenic in vitro.

**Cell mutation assays**

No increase in mutants was found using 4 h exposure of S49 mouse lymphoma cells to ethanol in the presence and absence of S9, using resistance to dexamethasone, 6-thioguanine and ouabain for mutant selection (Friedrich and Nass, 1983). In the L5178Y mouse lymphoma assay ethanol was negative at
concentrations up to 35.9 mg/ml (780 mM) (Amacher et al., 1980), a very high concentration, well above the maximum recommended for this type of test.

Ethanol was one of 50 compounds tested by Wangenheim and Bolecsfoldi (1988) in a study designed to test the conditions under which false positive results may be generated by the mouse lymphoma assay. Ethanol induced a small but statistically significant increase in mutants at several concentrations; 4.2 and 34 mg/ml (91 and 739 mM) without S9 and 24 mg/ml (522 mM) with S9. In no case was the mutant frequency doubled. Greater effects were obtained with similar concentrations of urea, DMSO, glucose and sodium chloride. The authors concluded that a maximum concentration of 20 mM was adequate to detect genotoxins and that higher concentrations may give false positive results. On this basis ethanol was clearly negative in this assay.

At Safepharm Laboratories ethanol solvent controls [usually 8 mg/ml (174 mM) ethanol] have shown no mutagenic effect either with or without metabolic activation in the mouse lymphoma cell mutation assay (see Appendix, Table AIII). The results of gene mutation assays with ethanol are universally negative.

**Micronucleus assays in vivo**

Ethanol had no effect on micronucleus incidence in the bone marrow when administered in the drinking water to rats at 5% ethanol (Korte et al., 1980) or to mice at up to 40% for 27 days (Chaube et al., 1977). In the study of Chaube et al. the highest dose resulted in some compound-related mortality and was therefore clearly above the maximum tolerated dose, recommended as the top dose for micronucleus studies in the current OECD guidelines (OECD, 1997, Guideline no. 474).

There have been two reports of positive in vivo micronucleus assays. Baraona et al. (1981) fed rats for 6 weeks with a diet in which ethanol constituted 36% of the calories (ethanol intake reported to be 12–16 g/kg/day). Compared with paired controls, the incidence of micronucleated bone marrow erythrocytes was increased from 0.95 to 1.30% in polychromatic erythrocytes (PCE) and from 0.67 to 0.84% in normochromatic erythrocytes (NCE). These differences were statistically significant, but are only marginal. They were associated with a decrease in the number of nucleated cells and an increase in the proportion of nucleated cells that were in mitosis in the bone marrow. The authors suggested that an effect on the mitotic spindle might be responsible for the observed effects. The presence or absence of centromeres in the micronuclei induced by treatment was not investigated. Small increases in micronuclei have been obtained after treatment of mice with erythropoietin, suggesting that effects on the rate of cell division in the bone marrow can lead to micronucleus formation, possibly due to errors in the processes of enucleation or differentiation of erythrocytes (Yajima et al., 1993).

Significant, but not dose-related, increases in micronuclei were reported by Badr et al. (1977) in mice injected i.p. on two consecutive days with 0.3 ml of 20–60% ethanol (0.62, 1.24 and 1.86 g/kg). However, the data show several inconsistencies that indicate that they may not be valid. The numbers of observed micronuclei are presented in both tabular and graphical form but the data appear to be inconsistent between the two forms of presentation. Also, the tabulated data report a control group mean micronucleus frequency of 4.63% in NCE and 5.6% in PCE. These values are 20–50 times the typical frequency seen in laboratory mice used in standard regulatory tests. This difference indicates that either the mice used in this study were grossly abnormal in this respect or that there may have been a problem with the technical quality of the slides or the slide scoring method used. The data also indicate similar frequencies of micronuclei in NCE and PCE. This cannot have occurred as a result of exposure to ethanol because the kinetics of the development of NCE from PCE does not allow for the observation of equivalent numbers of micronucleated erythrocytes of the two types at the single time point of 30 h that was used. The authors explain the inverse dose–response relationship as possibly resulting from excessive toxicity in the bone marrow. This could have easily been tested by a determination of the PCE/NCE ratio but the data were either not generated or not presented. Because of the apparent errors in this report, and the deviations from internationally recognized procedures, it is considered that a reliable conclusion cannot be drawn from its data.

Ethanol at its maximum tolerated dose level (2000 mg/kg) was used as the vehicle in a standard micronucleus test using the oral dose route at Safepharm Laboratories, conducted to GLP, and no increase in micronucleus frequency above expected levels was observed (see Appendix, Table AVI).

Overall, there is no convincing evidence that ethanol induces micronuclei in the bone marrow of rodents.

**Chromosome aberration tests in vivo**

No acute chromosome aberration tests have been reported. There have been several reports of sub-acute studies with negative findings. Male Wistar rats were given 10 or 20% ethanol in the drinking water for 3 or 6 weeks and no chromosome aberrations were found in the bone marrow or peripheral blood lymphocytes (Tates et al., 1980). In Chinese hamsters no aberrations were found in the bone marrow after exposure to ethanol in the drinking water at 10% for 9 weeks (Korte et al., 1979) or at 20% for 12 weeks (Korte et al., 1981a). No aberrations were found in lymphocytes after exposure of Chinese hamsters to 10% ethanol in the drinking water for 46 weeks (Korte et al., 1981b).

**Dominant lethal assay**

There is considerable controversy surrounding the possible activity of ethanol in the dominant lethal assay. A high level of dominant lethality in mice has been reported in one series of studies (Badr and Badr, 1975; Badr et al., 1977). In one experiment eight male mice treated with ethanol (1.24 g/kg body wt) by intubation on three consecutive days were mated sequentially to one female mouse per week. On the third week of mating only was there a marked reduction in the mean litter size of the ethanol group. No examination of the uterine contents was undertaken and yet this was taken as evidence of post-implantation loss due to dominant lethal mutations. The method that was used to calculate the dominant lethal index is only appropriate for very potent mutagens (Kratochvila, 1978). Furthermore, non-genetic effects can result in a reduction in mating frequency, fertilization frequency or implantation frequency (Anderson, 1984); this possibility was not considered by the author. In a second experiment, where mice were treated with either 1.24 or 1.86 g ethanol/kg, the uterine contents were examined on gestation days 13–15. The authors report significant increases in the frequency of dead implants and of the dominant lethal index at the second and third mating time points, i.e. days 4–8 and 9–13. This
response does not correlate with the previous experiment, in which the effect was seen only at the 14–17 days time point. The magnitude of the response reported for the second experiment is roughly equivalent to a dose of 400 R of X-rays or 300 mg/kg ethyl methanesulphonate, both of which are incontrovertible and extremely potent mutagens. Furthermore, from a study deliberately designed to reproduce the effects reported by Badr and co-workers (Badr and Badr, 1975; Badr et al., 1977), but using i.p. injection rather than intubation, Rao et al. (1994) concluded that ethanol did not have a significant dominant lethal effect but caused some pre-implantation loss, which might be due to an effect on the fertilization capacity of sperm. They investigated the effects of ethanol (~1.26 g/kg/day for 3 days) on the outcome of matings at three time points after treatment (days 1–4, 5–8 and 9–12). Data were reported for 83 treated Swiss mice and 71 controls. There was a marked reduction (by 34 and 30%) in the number of pregnant females at the first two mating times in the treated group and a significant decrease in total and live implants in the second mating. There was no increase in dead implants from the first two matings and only a small increase at the third mating, which was statistically significant \((P < 0.05)\) when compared with the concurrent controls but not the overall control frequency. No dominant lethal effect was found in two other experiments using CBA and C57BL6 mice chronically exposed to ethanol in the drinking water.

Negative results were also obtained in a large collaborative study in which mice were exposed by intubation to the maximum tolerated dose of ethanol (0.64 g/kg) and to 0.16 g/kg and mated for 8 weeks (James and Smith, 1982). The data, from three laboratories, showed no evidence of dominant lethality for ethanol. The protocol design used was comprehensive and compliant with OECD test methods. In view of these negative findings, the data of Badr and co-workers must be treated with caution.

In other studies in mice ethanol has been administered as part of a liquid diet. No dominant lethal effect was found when ethanol was included in a liquid diet as 20 or 30% of dietary calories for 4 weeks (Randall et al., 1982). Measured ethanol intake in this study was 24–30 g/kg/week for the 30% diet and 14–17 g/kg for the 20% diet and mean blood levels were 80 and 57 mg/dl, respectively. In a similar study using ethanol at 28% of dietary calories 5 week exposure of mice decreased testicular weight, reduced fertility and increased pre-implantation losses, fetal mortality and mutation index (Berryman et al., 1992). In this study ethanol intake was estimated as 22–25.5 g/kg/day.

In rats 15 days exposure to ethanol, increasing to 58% of dietary calories (estimated ethanol intake 7.2–14.4 g/kg/day), resulted in an increase in early abortions, which might be interpreted as a dominant lethal effect (Klassen and Persaud, 1976). However, only six pregnancies were examined in the ethanol treatment group and the males had been treated chronically with ethanol, such that their body weight was reduced by 40% after 30 days and their reproductive performance was reduced to a level that affected the ability of the study to generate adequate data for evaluation. Increases in the frequency of dead implants were also found when male rats were treated with 20% ethanol in drinking water for 60 days prior to mating (Mankes et al., 1982). Histological examination of the ethanol-treated rats revealed significant, and in some cases gross, pathological changes in the testes and effects on the litters could have resulted indirectly from toxicity to the testes. In contrast, Chauhan et al. (1980) found no effect of exposure of rats via the drinking water (30% ethanol) for 5 weeks. These were all small studies (group sizes of \(\leq 10\)) and the data must therefore be viewed with caution.

The majority of studies cited above can be criticized on the grounds of inadequate numbers of animals or on the methods used to score or evaluate the incidence of early or late fetal deaths. In most cases no distinction was made between early and late deaths. In many cases interpretation of the results was compromised by effects on fertility at the very high ethanol doses used. The most satisfactory test is the inter-laboratory study performed to OECD guidelines (James and Smith, 1982), which gave a negative result in mice. It may therefore be concluded that ethanol is negative in the dominant lethal assay in male mice.

A study of dominant lethal effects in female mice treated with a single oral dose of ethanol (4 g/kg) at pro-oestrus gave negative results (Machemer and Lorké, 1975). Treatment of female mice with a similar dose 1–2 h after mating (Washington et al., 1985) caused an increase in late deaths which, it was suggested, might be due to aneugenicity (see below).

**Conclusions from standard assays**

There are some clear inadequacies in the available data on the genotoxicity of ethanol. In only a few instances have tests been conducted to a standard which would now be regarded as satisfactory for defining a positive or negative response. The negative results of a number of bacterial mutation assays appear to be reliable but studies of chromosome aberration, though generally negative, can be criticized, particularly those which did not include an exogenous metabolic activation system. Of the mammalian cell mutation assays only one, the most extensive study, revealed a weak mutagenic effect in L5178Y mouse lymphoma cells, but only at very high ethanol concentrations. When due regard is taken of possible artefacts this study can be regarded as negative. Experience with ethanol as a solvent control suggests that it is not clastogenic or mutagenic \textit{in vitro}, either in the absence or presence of S9. \textit{In vivo} tests for chromosome aberrations in both rats and Chinese hamsters have given negative results. The results of the micronucleus and dominant lethal assays are variable, but the most extensive and better quality studies are negative. Using a weight of evidence approach we conclude that ethanol does not induce dominant lethality in assays using standard regulatory methodologies. Interpretation may be confused by the toxic effects of ethanol on the male reproductive system, which may influence the fertilizing capacity of sperm by non-genetic mechanisms.

Overall, there is very little evidence to suggest that ethanol is genotoxic in somatic cells and that, at worst, it may have a very limited capacity to induce genetic changes \textit{in vivo}, under very specific circumstances and at very high doses, which would only be achievable in humans by deliberate oral ingestion. No report has been found of an \textit{in vivo} DNA repair test on ethanol and, in view of the fact that a great deal of ethanol metabolism occurs in the liver and that this organ is damaged by chronic ethanol abuse, a liver unscheduled DNA synthesis assay would be valuable.

It is of interest to assess what the outcome might be if ethanol were a new chemical substance, without any pre-existing regulatory (GLP) or non-regulatory data, and was tested by a chemical manufacturer to meet the requirements...
of the EC mutagenicity testing strategy in order to notify the chemical for use in the EU. Assuming that a full notification to Annex VIIA of the seventh amendment of the dangerous substances directive was required then the initial test to be performed would be a bacterial mutagenicity test according to the EU B10/OECD 471 test guidelines. The result of this test would be negative, as shown by the published studies, the reported compatibility of ethanol as a solvent in bacterial assays and extensive vehicle control data.

Following a negative bacterial assay result, the strategy requires an in vitro chromosome aberration test in mammalian cells. For such assays it is typical to use either primary human lymphocytes or a rodent cell line such as CHO or CHL. A study performed according to the EC B14/OECD 473 test guidelines would use a maximum dose level of 5 mg/ml or 10 mM, whichever is the lower. Thus, for ethanol the 10 mM (0.46 mg/ml) dose level would be selected. However, even if 5 mg/ml were used, the published data would indicate a negative result in the absence of metabolic activation. Data from many studies using ethanol as a vehicle control show a lack of clastogenicity either with or without metabolic activation, although a fully compliant chromosome aberration test in vitro has not been published.

When the supply of a chemical reaches Level 1 (100 t.p.a., 500 t cumulative), or earlier if extensive human exposure may occur, then a third in vitro mammalian cell assay would be required to EC B17/OECD 476 test guidelines, either the mouse lymphoma or HPRT assay. Again, a negative result could be predicted from the published data and vehicle control data.

At Level 2 (100 or 1000 t.p.a.) a fourth test may be required and would be likely to be a micronucleus test in the mouse bone marrow according to the EC B11/OECD 474 test guidelines. The maximum dose level recommended for this study type is 2 g/kg and this has been shown to be negative when used as a solvent control. None of the published studies is of a sufficiently high standard to confirm this finding, but negative results were found in all three studies using mice treated orally.

In conclusion, a new chemical substance identical to ethanol would probably be notified in the EU as a non-genotoxic substance without any adverse data or structural concerns and no labelling requirements would be required.

**Supplementary information on genetic effects**

A great many studies have been conducted on the effects of ethanol in systems that are not routinely used in genotoxicity test batteries but which provide useful additional information. **DNA adduct formation**

When rats were given 5% ethanol in the drinking water for 8 months (intake 4.3 g/kg/day) no increase in DNA adducts was found by 32P-post-labelling in liver, lung, oesophagus or heart (Izzotti et al., 1998). Adducts induced by cigarette smoke in the heart and lungs were significantly increased by ethanol consumption and were increased in the oesophagus only after combined cigarette smoke and ethanol treatment. Several possible mechanisms for the potentiation of DNA damage by ethanol were suggested, including a solvent effect and alterations in metabolic enzymes, but this study shows that ethanol itself does not cause DNA adducts.

**Mutation in Drosophila, fungi, yeast and plant cells**

In *Drosophila* negative results have been obtained using the sex-linked recessive lethal assay (Vogel, 1972; Vogel and Chandler, 1974; Creus, 1983; Vogel et al., 1983; Woodruff et al., 1984). In *Aspergillus nidulans* tests for chromosome damage and mutation were negative (Morpurgo et al., 1979; Gualandi and Bellincampi, 1981; Kafer, 1984). In *Saccharomyces cerevisiae* ethanol induced petite mutations (Bandas and Zacharov, 1980; Bandas, 1982; Cabeca-Silva et al., 1982; Hamada et al., 1985) but did not induce gene conversions (Barale et al., 1983). The significance of induction of petite (mitochondrial) mutations is not clear. Ristow et al. (1995) demonstrated single-strand DNA breaks in a repair-deficient strain of *S. cerevisiae* but not in wild-type cells.

Tests for chromosome damage in plant cells have shown uniformly positive results for ethanol, which has been shown to induce micronuclei, chromosome damage and SCEs in the cells of a variety of plants (Michaelis et al., 1959; Schubert et al., 1979; Ma et al., 1984; Cortes et al., 1986; Zhang et al., 1991). This suggests that plant cells are either more sensitive than mammalian cells to the clastogenicity of ethanol or that they have a metabolic capacity which converts ethanol to an active clastogen. The latter explanation is supported by the observation that plant extracts can mediate chromosome damage induced by ethanol in mammalian cells in vitro (Darroudi and Natarajan, 1987) but it is not clear whether plant cells have a qualitatively or quantitatively different metabolic activity compared with mammalian cells.

**Sister chromatid exchange (SCE)**

The majority of tests for SCE in cultured cells have given negative results with ethanol, in human lymphocytes (Obe and Ristow, 1979; Jansson, 1982; Konigstein et al., 1984; Banduhn and Obe, 1985), human lymphoid cells (Sobti et al., 1982, 1983), CHO cells (Obe and Ristow, 1977; Schwartz et al., 1982; Darroudi and Natarajan, 1987) and mouse kidney fibroblasts (Garcia Heras et al., 1982). However, most of these studies did not use an exogenous metabolic activation system; only in the case of the human lymphoid cells was ethanol found to be negative both in the presence and absence of S9. SCE induction has been reported in only one study with human lymphocytes in the absence of S9 (Alvarez et al., 1980a). In studies where metabolic activation was used with CHO cells, one detected a weak effect without S9 which was increased in its presence (De Raat et al., 1983) and another showed a positive effect with both rat liver S9 and an extract from plant cells (Takehisa and Kanaya, 1983). In contrast, SCE induction by ethanol has been reported in the majority of published in vivo studies, although the effects have generally been small (less than a doubling of the background frequency). In the mouse SCE was induced in the bone marrow when ethanol was given in the drinking water at 10 or 20% for 3–16 weeks (Obe et al., 1979) or injected i.p. at 0.6–2.4 g/kg (Pina Calva and Madrigal-Bujaidar, 1993). SCE induction has also been observed after ethanol treatment in mouse peritoneal lymphocytes (Hirai and Nakaya, 1988), spleen cells (Zhang et al., 1991), spermatogonia (Hirai, 1988) and cells of embryos exposed in utero (Alvarez et al., 1980b; Czajka et al., 1980). Negative results were obtained after dosing mice orally on four consecutive days with 10% ethanol (Nayak and Buttar, 1986). In the rat exposure via the drinking water to 10 or 20% ethanol for 3–6 weeks caused an increase in SCE in lymphocytes but not in bone marrow (Tates et al., 1980). SCE was not increased in the bone marrow of Chinese hamsters given 10% ethanol in the drinking water for 46 weeks (Korte et al., 1981b) or 20% ethanol for 12 weeks (Korte et al., 1981a).
The higher proportion of positive reports of SCE induction by ethanol in vivo, compared with in vitro, might be taken to suggest that its effect is indirect, possibly involving metabolic conversion to an active form (possibly acetaldehyde). If this has a weak genotoxic effect, it might be more readily detected by the SCE assay than by chromosome aberration tests. However, at the high dose levels used it is also possible that disturbance of the metabolism or nutritional status of the animal was responsible for SCE induction. The exact mechanism of SCE formation, and its significance as a genotoxic end point, is still unclear. Increases in SCE frequency have been found in the bone marrow of malnourished and starved rats, associated with a decrease in cell proliferation rate (Veena and Murty, 1994). SCE frequency has also been found to be affected by hormonal status (Joseph-Lerner et al., 1993).

Chromosomal effects in alcoholics
A number of studies have shown increased levels of chromosome aberrations and SCE in the lymphocytes of alcoholics (reviewed in Obe and Anderson, 1987). The number of exchange-type aberrations in alcoholics was approximately three times that found in non-alcoholics and SCE frequency was increased by a smaller proportion, generally ~20%. These effects disappeared after a period of abstinence from alcohol consumption.

These data suggest that alcoholism may cause chromosome damage in humans. However, it cannot be concluded that the direct action of ethanol on chromosomes is responsible for these effects. Alcoholism involves a general disturbance to dietary habits, nutritional status and metabolism, exposure to agents other than ethanol in alcoholic beverages and, possibly, increased exposure to other potential mutagens (e.g., cigarette smoke). The significance of the increase in SCE is, as discussed above, open to debate.

Non-disjunction and aneuploidy
Two studies of the chromosomes of lymphocytes from alcoholics have found an increased incidence of aneuploidy (Mitelman and Wadstein, 1978; Kucheria et al., 1986) and an association between drinking and aneuploidy in sperm was found in a study of young men (Robbins et al., 1997).

Induction of aneuploidy by ethanol has also been reported in the germ cells and embryos of rodents. In male mice treated with ethanol aneuploidy was increased in spermatocytes (Alvarez, 1983; Hunt, 1987) and in post-implantation embryos sired by ethanol-treated males (Barilyak and Kozachuk, 1981). These studies may be criticized on the grounds of inadequate numbers of cells evaluated, high background frequencies and also the possibility of technical artefacts, particularly where the increase in aneuploidy is largely due to increased hypoploidy. Chromosome dissociation during meiosis was reported by Hirai (1988), but this end point is a poor predictor of aneugenic potential (ECETOC, 1997). No aneugenic effect was found in the spermatogonia or meiotic cells of Chinese hamsters (Daniel and Roane, 1987).

An increased incidence of aneuploidy was found in mouse eggs treated with ethanol in vivo and at the first cleavage and morula stages of embryos when female mice were treated with ethanol at around the time of mating (Kaufman, 1983). Ultrastructural studies suggested that spindle disturbances, possibly mediated by disruption of calcium homeostasis, might be responsible for ethanol-induced non-disjunction, leading to aneuploidy (O’Neill et al., 1989). The ethanol doses used in these studies were ~3-4 g/kg. It was speculated that ethanol-induced non-disjunction might be responsible for a proportion of spontaneous abortions in humans (Kaufman and Bain, 1984).

Washington et al. (1985), using a similar treatment of female mice with ethanol 1–2 h after mating, found a significant increase only in hypoploidy, which may be caused by chromosome loss during cytological preparation. However, they also found a significant increase in late deaths in a dominant lethal test using the same treatment schedule. Treatment of female mice with ethanol shortly before or after mating has been shown to increase the number of fetal resorptions, late deaths and anatomic abnormalities (Soltes et al., 1996). These effects could be due to an aneugenic effect during meiosis or embryonic cleavage stages but convincing evidence for this proposal is lacking and alternative non-genotoxic mechanisms are feasible.

Chronic exposure of rats to ethanol in utero was found to cause aneuploidy in fetal liver cells (Kozachuk and Barilyak, 1982) but intra-amniotic injection of ethanol on gestation day 13 had no effect (Barilyak and Kozachuk, 1983).

Evidence for an aneugenic effect of ethanol also comes from genotoxicity tests on Drosophila and Aspergillus. In Drosophila a test for non-disjunction was positive (Rey et al., 1992). Experiments with germinating conidia of A. nidulans have shown that ethanol causes chromosome malsegregation, resulting in aneuploidy and polyploidy (Harsanyi et al., 1977; Morpurgo et al., 1979; Gualandi and Bellincampi, 1981; Kafer, 1984; Crebelli et al., 1989).

It is interesting to speculate on the possible involvement of an aneugenic effect of ethanol in the results of the dominant lethal assays (exposure of males) discussed earlier. In principle, non-disjunction during spermatogenesis could lead to aneuploidy, which might cause fetal death. However, the dominant lethal effect reported by Badr et al. (1977) was seen at mating times of 4–17 days after treatment and would only be consistent with an effect on post-meiotic spermatids or spermatozoa. It is difficult to envisage a mechanism by which an effect on microtubules could result in lethal mutations at this late stage. Furthermore, the effect was not reproducible and therefore may not be real.

Micronucleus induction may result from either chromosome breakage or loss of whole chromosomes. It is possible, therefore, that the two micronuclear assays reported as positive were detecting an aneugenic effect; micronuclei were not examined for the presence of centromeres. An aneugenic effect during mitosis or meiosis might be invoked to explain some of the positive results obtained after chronic ethanol treatment but does not help to resolve the conflict between positive and negative findings.

Discussion
Overall, there is little evidence to suggest that ethanol is a direct acting mutagen, a fact which would not be unexpected in view of the chemical structure and relative unreactivity of the ethanol molecule. The weak, and often elusive, positive results in various systems could be explained in a number of ways.

Acetaldehyde as a mutagenic metabolite
The genetic effects observed in some studies might be due to an ethanol metabolite, presumably acetaldehyde, which is not generally formed in significant amounts in most in vitro systems and which is produced only in small amounts in specific tissues in vivo. This might explain why genetic effects
in vivo appear to be detectable with ethanol only at very high dose levels, by only some routes of administration and only in certain animal species and strains.

SCE can be induced in human lymphocytes in vitro by acetaldehyde (Obe and Ristow, 1977) or if ethanol treatment is conducted in the presence of alcohol dehydrogenase (Obe et al., 1986). This suggests that acetaldehyde may be the agent responsible for SCE induction by ethanol in vivo in animals and in alcoholics. The same mechanism can be invoked to explain the increase in chromosome aberrations found in the lymphocytes of alcoholics. There is little evidence of a clastogenic effect of ethanol in mammalian cells in vitro but these studies generally did not use a metabolic activation system which might have modelled acetaldehyde formation by the liver. However, animal studies have failed to demonstrate chromosome aberrations after ethanol exposure. If acetaldehyde is responsible for chromosome damage the balance between its rate of formation by alcohol dehydrogenase and its conversion to acetate by aldehyde oxidase will be of crucial importance. It is uncertain whether S9 will adequately model the metabolism of ethanol.

Acetaldehyde itself appears to be negative in the Ames test but has been found to induce SCE and chromosome aberrations in cultured mammalian cells (Obe and Anderson, 1987). It was also positive in the mouse lymphoma mutation assay (Wangenheim and Bolcsfoldi, 1988). However, there have been very few in vivo studies. Obe et al. (1979) found a moderate increase in SCE in the bone marrow of mice but only two animals were used and 50 metaphases per animal analysed. Korte et al. (1981b) reported an increase in SCE in the bone marrow of hamsters given 0.5 mg/kg acetaldehyde i.p.

It is of interest that acetaldehyde can cause changes in tubulin polymerization in hepatocytes (Lieber et al., 1987), an effect which suggests that it could also cause spindle disturbances and aneuploidy. However, high concentrations were required for an effect and it seems unlikely that sufficiently high concentrations could occur in extra-hepatic tissues.

General metabolic disturbances
Changes in liver enzymes or hormone levels caused by very high ethanol doses may have an indirect effect in vivo, possibly involving induced micronutrient deficiency or oxidative stress. It is clear that many studies on ethanol have been designed to investigate the possible effects of alcohol abuse and, in most cases, effects have been measured in vivo in animals showing clear signs of chronic toxicity. High doses of ethanol have marked effects on the metabolism of the liver, affecting not only the metabolism of ethanol itself and that of other xenobiotics but also increasing the levels of oxygen radicals and depleting sulphhydril compounds (Kurose et al., 1996; Lieber et al., 1987). The significance of these disturbances is that at high doses genotoxic agents may be formed which would not be formed, or not released into the circulation, at lower doses; there may be a threshold for genotoxicity in this case. In this context it is pertinent to note that endogenous ethanol is found in human blood at concentrations of 0.1–0.6 mg/l (Ostrovsky, 1986; Sprung et al., 1981). The source of this ethanol is uncertain but appears to be physiological.

Physico-chemical effects
The physico-chemical properties of ethanol, particularly its activity as a solvent, may result in disturbances to cellular structures other than DNA. In view of the many reports of aneuploidy induction in a variety of systems, it seems that the spindle could be a sensitive target. Ethanol, acting by damaging membranes and causing ion imbalance, may have a direct effect on spindle function. The solvent properties of ethanol and its effects on liver enzyme activities (and possibly those of the gut microflora) could conceivably lead to the increased formation of mutagens derived from dietary constituents.

Conclusions
The available data derived from studies on ethanol using standard genotoxicity test methods are incomplete. There is clear evidence that ethanol is not a bacterial or mammalian cell mutagen but published in vitro assays for chromosome aberration have generally not included exogenous metabolic activation. However, evidence from the use of ethanol as a vehicle control suggests that ethanol is not clastogenic in vitro.

In some of the rodent assays in vivo there is genuine conflict in the results of different studies which cannot be explained by obvious protocol deficiencies. The reported tests for chromosome aberration in vivo are all negative and only a minority of micronucleus tests have given positive results. Data for dominant lethal assays are almost equally divided between positive and negative, although the inter-laboratory study performed to OECD guidelines was negative. There is some, limited evidence that ethanol induces SCE in vivo and can also act as an aneugen at high doses.

Many of the in vivo studies reviewed were clearly designed to model the effects of alcoholism; animals were treated with very high doses, sometimes for long periods. The results may therefore be affected by disturbances of metabolism giving rise to secondary genetic effects. Few of the published studies conform to currently accepted standards for mutagenicity testing of industrial chemicals and those which do show that ethanol is not mutagenic.

There is no convincing evidence that ethanol is a genotoxic hazard if the same criteria are applied to its assessment as would be applied to other industrial chemicals. There are numerous reasons for concern about excessive consumption of ethanol in beverages and some degree of genotoxicity may be included, but it is not relevant to the orders of magnitude lower levels which are obtainable by either inhalation or dermal exposure in the workplace.

On the basis of the available evidence, we conclude that ethanol should not be classified as a mutagen.

Acknowledgement
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References


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**Is ethanol genotoxic?**
Appendix. Ethanol as a vehicle control: Safepharm Laboratories in-house data

Bacterial mutation tests
Ethanol has been reported as being compatible with the Salmonella/microsome test at 200 μl/plate in the plate incorporation assay and up to 100 μl/plate in the pre-incubation assay (Maron et al., 1981). At Safepharm Laboratories ethanol has been used as one of the validated vehicle controls for more than 10 years. The typical dose volume used is 100 μl/plate, which is equivalent to 79 mg/plate, or ~16 times the normal maximum recommended dose level of 5 mg/plate used in regulatory mutagenicity tests. In 1998 it was used as the vehicle for 18 test materials, which was ~5% of the total number of studies completed in that year. The mean, minimum and maximum frequencies of revertant colonies for the ethanol vehicle control plates were all comparable to the 1998 vehicle control history profile for all vehicle controls used at Safepharm Laboratories in 1998. The data for the two history profiles are given in Tables A1I and A1II.

L5178Y mouse lymphoma assays
Ethanol was used as the vehicle control in only one study during 1998. At a dose volume of 100 μl/10 ml (1%) ethanol was non-toxic and non-mutagenic to L5178Y cells. This concentration is equivalent to 7.9 mg/ml, which is ~1.6 times the maximum recommended dose level of 5 mg/ml. However, in molar terms 1% is equivalent to 170 mM, which is 17 times the maximum recommended dose level of 10 mM suggested by the OECD test guidelines. The vehicle control mutation frequencies for the study using ethanol were 98.90, 79.56, 134.44 and 125.59×10⁻⁶ for the two without activation and two with activation experiments, respectively. These values are within the range of mutation frequencies found for controls using other vehicles; the data from 20 experiments conducted immediately prior to the test using ethanol are shown in Table AIII.

Chromosome aberration tests in vitro
Ethanol is used infrequently as a vehicle control in chromosome aberration tests in vitro. However, in the last 10 years it has been used in a sufficient number of studies to demonstrate that it is not clastogenic to either human lymphocytes or to Chinese hamster lung cells (CHL). As in gene mutation assays, the dose volume of 1% (100 μl/10 ml) exceeds the maximum recommended dose levels suggested by the OECD test guidelines. The vehicle control data for the studies using ethanol are compared with historical control data for 1997 in Tables AIV and AV. In both cell types the mean values for ethanol controls were slightly higher than the overall control means but the maximum values were similar in all cases.

Bone marrow micronucleus studies in the mouse
Ethanol was used on only one occasion to formulate a particularly difficult test material. The vehicle used was actually 20% ethanol in arachis oil, dosed at 10 ml/kg via the i.p. route. Therefore, the dose of ethanol received by each animal was non-toxic and non-mutagenic to L5178Y cells. This concentration is equivalent to 7.9 mg/ml, which is ~1.6 times the maximum recommended dose level of 5 mg/ml. However, in molar terms 1% is equivalent to 170 mM, which is 17 times the maximum recommended dose level of 10 mM suggested by the OECD test guidelines. Three vehicle control groups were used in the study; at the 24, 48 and 72 h time points. The frequency of PCE with micronuclei was 1.3, 1.2 and 0.8 per 1000 cells. All three values are within the current historical range for the micronucleus test at Safepharm Laboratories; data from 60 recent studies are given in Table AVI.

| Table A1. Ames test history profile: vehicle control values 1998 |
|-------------------|---------|---------|---------|---------|---------|
| Strain            | TA100   | TA98    | TA1535  | TA1537  | TA102   |
|                   | −S9     | +S9     | −S9     | +S9     | −S9     | +S9     |
| No. of values     | 342     | 339     | 337     | 340     | 341     | 331     |
| Mean              | 124     | 125     | 31      | 36      | 26      | 18      |
| SD                | 16      | 16      | 6       | 6       | 5       | 4       |
| Min.              | 73      | 81      | 15      | 22      | 12      | 10      |
| Max.              | 173     | 170     | 45      | 54      | 38      | 37      |
|                   | 20      | 22      | 20      | 23      | 200     | 285     |

| Table A2. Ames test history profile: ethanol control values 1998 |
|-------------------|---------|---------|---------|---------|---------|
| Strain            | TA100   | TA98    | TA1535  | TA1537  | TA102   |
|                   | −S9     | +S9     | −S9     | +S9     | −S9     | +S9     |
| No. of values     | 19      | 19      | 20      | 20      | 18      | 17      |
| Mean              | 118     | 131     | 35      | 37      | 30      | 30      |
| SD                | 6       | 13      | 4       | 1       | 6       | 3       |
| Min.              | 113     | 121     | 32      | 36      | 26      | 28      |
| Max.              | 122     | 140     | 38      | 37      | 34      | 32      |
|                   | 2       | 2       | 2       | 2       | 290     | 304     |
Is ethanol genotoxic?

Table AIII. L5178Y mutation test: vehicle control data for 20 experiments

<table>
<thead>
<tr>
<th></th>
<th>Without activation</th>
<th>With activation</th>
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</thead>
<tbody>
<tr>
<td><strong>Mean</strong></td>
<td>95.69</td>
<td>106.63</td>
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<tr>
<td><strong>SD</strong></td>
<td>32.09</td>
<td>25.68</td>
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<tr>
<td><strong>Max.</strong></td>
<td>185.98</td>
<td>148.71</td>
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<tr>
<td><strong>Min.</strong></td>
<td>31.93</td>
<td>60.31</td>
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Table AIV. Chromosome aberration test in human lymphocytes: vehicle control data

<table>
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<tr>
<td></td>
<td>−S9</td>
<td>+S9</td>
</tr>
<tr>
<td><strong>No. of values</strong></td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td><strong>Mean</strong></td>
<td>1.5</td>
<td>0.7</td>
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<tr>
<td><strong>SD</strong></td>
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<td>0.8</td>
</tr>
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<td><strong>Max.</strong></td>
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<td>1.5</td>
</tr>
<tr>
<td><strong>Min.</strong></td>
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Table AV. Chromosome aberration test in CHL cells: vehicle control data

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<tr>
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</tr>
<tr>
<td><strong>No. of values</strong></td>
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<td>6</td>
</tr>
<tr>
<td><strong>Mean</strong></td>
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</tr>
<tr>
<td><strong>SD</strong></td>
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<tr>
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<td><strong>Min.</strong></td>
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Table AVI. Mouse micronucleus assay: vehicle control data

<table>
<thead>
<tr>
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<th>Frequency of micronuclei/1000 PCE</th>
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<td>24 h</td>
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<tr>
<td><strong>No. of studies</strong></td>
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<tr>
<td><strong>Mean</strong></td>
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<tr>
<td><strong>SD</strong></td>
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<tr>
<td><strong>Min.</strong></td>
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<tr>
<td><strong>Max.</strong></td>
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