The Effect of Magnetic Field Exposure on Mutagenicity in the Transgenic R2λLIZ Rat Cell Line

by

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ABSTRACT

The possibility of a magnetic field (MF) mediated mutagenic effect was examined by detecting mutations in the *E.coli lacI* gene in a transgenic rat embryo fibroblast cell line, R2λLIZ. These experiments were motivated by the recently demonstrated MF induced stabilization of free radicals by chemical studies. Herein it is asked whether this phenomenon is detectable, in terms of mutation induction, in a cell culture system. Mutant frequencies were determined from untreated cells and cells exposed to the mutagens menadione, N-methyl-nitrosourea (MNU), or trenimon in the presence or absence of a three milliTesla 60 Hz magnetic field. Two complimentary exposure protocols were conducted; MF exposure following chemical pre-exposure, and MF exposure during chemical exposure. While it was observed that the chemicals are mutagenic (with menadione and trenimon causing a two-fold and MNU a six-fold induction over background), comparison of the mutant frequencies observed between the two experiments suggests that the magnetic field used in this study is neither mutagenic nor co-mutagenic, and therefore evidence for radical stabilization was not found. Mutational spectra for the exposure groups were ascertained, and no significant differences in spectra were found between the field exposed and control groups. Compared to untreated controls, however, it was found that menadione and MNU exposed cells have significantly higher proportions of G:C→A:T transitions at non CpG sites. Further, a disproportionately high number of G:C→T:A transversions were recovered from cells exposed to trenimon.

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CHAPTER I - INTRODUCTION

Electric and magnetic fields (EMF) have been implicated as a cancer risk by several epidemiological studies (reviewed by Schreiber *et al.*,1993; Feychting *et al.*, 1993; Matanoski *et al.*,1993; Loomis *et al.*,1994; Theriault *et al.*, 1994; and Sahl *et al.*,1993). The literature is far from unanimous in the association between cancer and exposure to electromagnetic fields. In the absence of unanimity within the scientific community, the possible health risks of magnetic fields remain the concern of a public most aware of the positive studies (Taubes, 1994). Molecular biologists have responded to the epidemiology by investigating various endpoints of EMF-mediated interaction *in vitro* and *in vivo*. Examples of these endpoints include: ATP modulated thymidine incorporation in tumourigenic cell lines (Colacicco *et al.*, 1983), DNA synthesis and cell survival (Cossarizza *et al.*, 1989; Rius *et al.*, 1985), specific enzyme activity (Litovitz *et al.*, 1991), chemically induced tissue damage (Cadossi *et al.*, 1992), DNA damage and the potentiation of oxidative stress (Scarfi *et al.*, 1993; Fairbairn *et al.*, 1994; Lai & Singh, 1997), skin tumour co-promotion in mice (Stuchly *et al.*, 1992; Rannug *et al.*, 1993, and reviewed by Holmberg, 1995), melatonin suppression (reviewed by Reiter, 1994), and many investigations on transcriptional activation (Goodman *et al.*, 1992 ; Blank *et al.*, 1992; Phillips *et al.*, 1993b; Goodman *et al.*, 1994a, 1994b, 1994c; Gold *et al.*, 1994; Libertin *et al.*, 1994). No clear mechanism of interaction between MFs and biological material has emerged. Certainly no single mechanism explains the suggested increased cancer risk. The same can be said about the medical uses of EMFs, which include the treatment of musculoskeletal disorders, myocardial and cerebral ischemia and chronic bone fractures, the induction

of nerve regeneration and wound healing, and the alteration of graft behaviour (reviewed by Basset, 1993). The diversity of these phenomena suggests that there may be multiple pathways of interaction between electromagnetic fields and biological tissues. Whether one of these possible pathways includes a role as a mutagen or co-mutagen is as yet unknown.

The process of mutagenesis is known to be involved in the production of human cancers. The alteration or loss of function of one or more genes essential to normal cell regulation, such as tumour suppressor genes, proto-oncogenes, growth factors and their receptors, DNA repair and xenobiotic-metabolizing genes, has become the paradigm in studies of the initiation of carcinogenesis (Gonzalez, 1995; Dumaz *et al.*, 1993; Gazdar, 1992; Kress *et al.*, 1992; Buetow *et al.*, 1992; Rady *et al.*, 1992; Holstein *et al.*, 1991; Simic *et al.*, 1986; Drake, *et al.*, 1983; Kleijer, *et al.*, 1970).

It has been demonstrated that a magnetic field can stabilize certain types of free radicals (Scaiano, 1995a; 1995b; 1995c; Scaiano *et al.*, 1994; Cozens & Scaiano, 1993; Okazaki *et al.*, 1988). Whether this effect is relevant or detectable in a cellular or biological system is uncertain. Several free radical species are capable of inducing genetic mutation. The superoxide anion radical is involved in the formation of the hydroxyl radical and singlet oxygen (Thor *et al.*, 1982). Both the hydroxyl radical and singlet oxygen can in turn cause 8-oxoguanine lesions (8-oxoG) (reviewed by Tchou & Grollman, 1993), and subsequently G:C→T:A transversion by misincorporation of adenine opposite the damaged base (Shibutani *et al*, 1990). Further, oxygen radical attack on the deoxyribose backbone of DNA can lead to base loss or strand breakage (Simic & Jovanovic, 1986). Given that some free radical species, such as the hydroxyl

and superoxide anion radicals, are capable of damaging DNA and hence of inducing genetic mutation, it is therefore possible that a magnetic field could potentiate genetic damage.

To investigate this possibility, experiments were conducted using the Big Blue® transgenic rat embryo fibroblast cell line, R2λLIZ. These cells contain 50-70 copies of an integrated lambda/*lacI* shuttle vector as a retrievable mutational target (Wyborski *et al.*,1995). In this system the *lacI* transgene is rescued from the fibroblast genomic DNA by λ-phage packaging extract and transfected into *lacI*⁻ *Escherichia coli* bacteria. Phage harbouring a mutant *lacI* gene form blue plaques, indicating the inability of the LacI repressor protein to inhibit β-galactosidase synthesis. The cells were exposed to the mutagens N-methyl-nitrosourea (MNU), 2-methyl-1,4-napthoquinone (menadione), or 2,3,5-triethyleneimino-1,4-benzoquinone (trenimon) in the presence or absence of a 60 Hz magnetic field (MF) at a flux density of 3 mT (1 T = 10^4 Gauss). Mutant frequencies were determined from cells pre-exposed to menadione (a free radical generating mutagen), to MNU (an alkylating mutagen), or to trenimon (a polyfunctional alkylating agent which can also cause oxidative stress and for which there is evidence of MF induced mutagenicity enhancement) subsequent to MF exposure. Frequencies were also determined from cells co-exposed to the abovementioned chemicals and the MF. This design allows for discernment between two possible routes of MF mediated enhancement of mutagenesis; an enhancement by a direct influence on the mutagens themselves, ie. by free radical stabilization, versus an enhancement by an indirect influence on the mutagenicity of the chemicals, for example by the alteration of membrane permeability (Goodman *et al.*, 1986). Sequence

analysis was performed on a subset of the mutations to compare the mutational spectrum for menadione, MNU, and trenimon to the spontaneous spectrum, and to screen for pronounced MF induced shifts in mutational spectra.

1.1 Outline and Rationale of Thesis

This project aims to investigate the potential influence of a MF on the process of mutagenesis and co-mutagenesis. The chemicals were chosen in part to test the radical stabilization hypothesis; menadione and trenimon being the 'test' chemicals and MNU the 'control' chemical; and in part for their proven mutagenic effects (Ono *et.al.*,1995 and Liu, *et al.*, 1994; Mangues, 1994; Obe & Beek, 1979). Trenimon was also chosen for study because of evidence suggesting that its genotoxicity may be enhanced by a MF (Rosenthal *et al.*, 1989). The project, however, is not limited to the testing of the stabilization hypothesis, since several other relevant questions, ie. is the field mutagenic or antimutagenic in the absence of chemical co-exposure? Are the observed effects likely to be resultant from a mutagenesis or a membrane transport enhancement? Are the chemicals alone mutagenic in this system ? What can be learned from the mutational spectra in terms of the mechanisms of mutagenesis of the chemicals?

While most work on MF-free radical phenomena have been demonstrated with photochemically generated, and carbon-centred, triplet radical pairs, the principles apply to radical pairs in general (Cozens and Scaiano, 1993; Scaiano, 1995b). Even as radical stabilization has been shown in enzymatic and physico-chemical studies (Harkins, *et al.*, 1994; Scaiano, 1994), it is yet to be determined whether this phenomenon is biologically significant or indeed whether it is detectable in existing

mutagenicity assays. The model of MF induced radical stabilization serves as the main hypothesis to be tested in the mutagenicity assay employed here.

The exposure protocols have been designed with the intention of defining possible causes of any detected mutagenic enhancements by a process of elimination. The first (staggered) exposure protocol used the design of Rosenthal and Obe (1989), wherein the MF acts only on the chemical which has remained in the cell after the 30 minute chemical exposure and wash steps. Since a MF mediated co-mutagenic potential may be stronger when the 30 minute chemical exposure exactly coincides with the field exposure, a second exposure protocol (concurrent) was also used. The concurrent exposure, however, introduces the possibility of MF interaction not only upon the chemical which has gained entry to the cell, but also upon the process of chemical entry into the cell. Conducting both these exposure regimes has the benefit of allowing the following question to be asked: Are differences in mutant frequency likely to reflect a direct MF influence on the chemical (such as radical stabilization) or instead some indirect influence on mutagenicity (such as changes to membrane permeability). Given the evidence in the literature on MF induced alterations in membrane hydrophobicity and thus permeability (Goodman *et al.*, 1986), it is necessary to employ both exposure protocols. The first experimental aspect of this thesis involves determining spontaneous and induced (in terms of chemical and/or MF exposure) mutant frequencies using both staggered and concurrent exposure regimes.

The second experimental component of this thesis involves the sequencing of subsets of mutations. Mutational spectra for menadione, trenimon, and for MNU were

determined and compared to the spontaneous spectrum. This information allows consideration of the mechanisms of mutagenesis of the chemicals within the R2λLIZ cells, and permits screening for any pronounced alterations in mutational spectra induced by the MF.

1.2 Electromagnetic Fields

The electromagnetic spectrum includes forms of energy ranging from extremely low frequency electromagnetic fields (ELF fields) to the high frequency forms of ionizing radiation such as hard X-rays and Gamma rays. Unlike these latter forms of radiation, ELF fields are non-ionizing; the energy associated with a 60 Hz 3 mT magnetic field is far less than the energy which constitutes a chemical bond. The magnetic field is spoken of as distinct from the electric field since the secondary (induced) field is very weak at extremely low frequencies (less than 100 kHz) (Deno and Carpenter, 1994). Furthermore, in this study the magnetic field is the primary factor since the electric field is shielded by the exposure apparatus.

Unlike an electric field, a magnetic field is not attenuated by biological tissue since the impedance of the magnetic field is the same for the tissue as it is for air (Deno and Carpenter, 1994). Since magnetic fields are not significantly perturbed by biological tissue one would expect the magnetic field employed here to be present within the nuclei of the R2λLIZ cells. There are expected to be induced electric fields within the tissue culture media (in conductive tissue in general) caused by the magnetic field (McCann *et al.*, 1993). While the induction of such fields was not modelled in the study, it is acknowledged that they could act as a confounder.

Magnetic fields attenuate with distance from the field source. Thus some of the epidemiological studies examining the link between electromagnetic fields and cancer have used proximity to powelines as a surrogate for EMF exposure. The ELF

magnetic field employed in this study is higher than those regularly encountered in residential or occupational settings (see Table 1.1).

Table 1.1 Electromagnetic Fields Encountered by Humans

Field Type	Exposure (milliTesla)*	Enivironment
ELF Magnetic Fields	0.00001-2.0	residential / occupational
	<10	extreme occupational/induction furnace workers
Static Magnetic Fields	0.01- 0.1	residential / occupational
	<100	occupational
	<4000	medical

Source: McCann, et al., 1993.

* 1mT = 10000 mG

1.3 Electromagnetic Fields and Cancer - The Epidemiology

Wertheimer and Leeper first correlated extremely low frequency EMF exposure and cancer incidence (in children) in 1979. Their work set a precedent for arguing that the magnetic rather than the electric component of the EMF to be responsible for the elevated cancer risks, since the electric field was thought to be shielded by the environment between the source of the fields (power lines) and the home. In this initial study, a relative risk of 2.2 was found for all cancers combined (95% confidence interval (CI): 1.6-3.1).

There are several recent epidemiological reports concerning residential and occupational EMF exposures, as well as numerous reviews (Schreiber *et al.*, 1993; Feychting & Ahlbom 1993; Matanoski *et al.*, 1993; Loomis *et al.*, 1994; Theriault *et al.*, 1994; and Sahl *et al.*, 1993). For example, by calculating historical MF exposures to children living near powerlines, Feychting & Ahlbom found a relative risk of 2.7 for childhood leukemia (CI 1.0-6.3). In general a weak association between cancer incidence (mainly leukemias, lymphomas and brain cancers) and MF exposure appears in the literature (Wertheimer and Leeper, 1979; Tomenius, 1986; Savitz, 1988; London, 1991).

In the epidemiology it does not always appear that people who have experienced the highest MF exposures are at the greatest risk: Matanoski *et al.* (1993) found that among the electrical occupations studied, cable splicers were at the lowest risk (odds ratio = 0.5) for leukemias even though they experience the strongest fields. Their study of telephone linemen did, however, reveal odds ratios of 2.4 (CI 0.7-9.0) and 6.6 (CI 0.7-58) for leukemias when latent periods of 10 and 15 years were used for exposure assessment. It

must also be noted that relative risks of unity have often been reported (Fulton, 1980; Myers, 1990; Savitz, 1988; Severson, 1988) and a protective effect has been reported in at least one study (Tomenius, 1986).

Comparison between these studies should be cautious since different methods of MF exposure assessment have been used. For example, wiring codes were used in several studies as a surrogate for exposure (Wertheimer and Leeper, 1979; Fulton, 1980), where other studies used spot measurements of MFs in the home (eg. Tomenius, 1986), or a combination of wire codes and spot measurements (Savitz, 1988; Severson, 1988; London, 1991). The association between MF exposure and leukemia is stronger in studies which use wiring codes than in those using spot measurements (reviewed by Feychting & Ahlbom, 1993). In a recent study which used spot measurements, for example, the odds ratio for acute lymphocytic leukemia (in children) was 1.24 (CI 0.86 to 1.79) at exposures of 0.200 μT or greater as compared with less than 0.065 μT (Linet *et al.*, 1997). Studies have also focused solely on electric fields. For example, Guenel *et al.* (1996) reported an association between electric field exposure and the incidence of brain tumours.

1.4 Electromagnetic Fields and Biological Systems

Most of the studies aimed at testing the biological effects of MFs have been conducted in the absence of mechanistic hypotheses of interaction between the field and the molecule, cell or organism. Exceptions include the studies relating MF exposure to regulation of the pineal hormone melatonin, as well as the few studies designed to test the potentiation of oxidative stress (Scarfi *et al.*, 1993; Fairbairn *et al.*, 1994). To put the present study in context, then, it is most important to focus on certain of the studies related to magnetic fields and genotoxicity. Also, many studies have been designed to test the ability of various MFs to regulate the transcription of certain genes; this area of research is first briefly reviewed to consider whether this phenomenon is likely to be a confounding factor in the present work.

The effect of MFs on transcriptional activity is an area of study almost as controversial as the debate over the health risks and possible benefits of magnetic fields. The approach taken in most expression studies has been to select candidate genes and to directly measure their transcriptional activity. Several investigations have focused on the levels of the nuclear proto-oncogene transcripts *c-myc*, *c-fos*, and *c-jun*. Other studies include cytoplasmic proto-oncogenes, growth factors, and tumour suppressor genes. The conclusions of these studies depend not only upon the cell and field type being examined, but also upon the exposure protocols employed.

Goodman *et al.* (1992) showed that *c-myc* transcript levels increase upon the exposure of human leukemic (HL-60) cells to a low frequency (60 Hz, 80 μT) sinusoidal MF. Goodman *et al.* (1994a) also showed a segment of the *c-myc*

promoter to be sufficient to activate the transcription of a chloramphenicol acetyl transferase (CAT) reporter gene in the presence of 60 Hz magnetic field (MF) in both mouse myeloma and human Hela cells. Interestingly, the mouse cells showed maximum CAT induction at a field strength of 80 μT & 100 μV/m after a 20 min exposure, whereas the maximum observed in the human cells occurred at only 8μT.

Although the earlier autoradiographic studies of Goodman *et al.* (1988) were interpreted as indicating that magnetic fields increased transcription at already active chromosomal loci (reviewed by Phillips *et al.*, 1993a), recent studies indicate a more complex process. Both the stimulation and the inhibition of specific transcripts have been observed upon MF exposure. The effect of EMF on transcription is not likely the consequence of a simple "switch-on" or "switch-off" pathway. It seems clear that the exposure protocol plays a critical role in the process. Phillips *et al.* (1992) exposed CEM-CM3 T-lymphoblastoid cells to a 1 Gauss (0.1 mT) 60 Hz sinusoidal MF and found an increase in *c-fos, c-myc* and protein kinase C transcription, and a decrease in that of *c-jun* when the cells were treated at a density of 5×10^5 cells/mL. At a density of 1×10^6 cells/mL, however, *c-jun* levels increased (as did the other three transcripts). Phillips *et al.* (1993b) also found that CCRF-CEM T-lymphoblastoid cells exposed to a 72 Hz/100 μT magnetic field for up to 28 hours showed a time dependent alteration of the *ras* proto-oncogene, p21, transcriptional activity. Specifically, during the first 6 hrs of exposure no change in p21 levels compared to controls was observed. During the 6-16 hrs period of exposure p21 levels decreased to 30% of control values. This was followed by a return to the normal

levels for the remainder of the exposure period. The authors conclude that the changed p21 protein expression is a result, at least in part, of changes in the levels of N-*ras* mRNA.

In the abovementioned studies and others of their kind, the magnetic field intensities that yield a transcriptional response are very low (<100 μT), with effects disappearing at higher flux densities. In contrast, the field intensity used in the present work is 3000 μT. Therefore there is little reason to believe that a transcriptional response would be experienced by the R2λLIZ cells. However, without specifically testing several transcripts for MF induced transactivation within these cells (at a field strength of 3 mT) it is still possible that transcriptional changes could confound the present study. While a MF mediated influence on transactivation seems plausible at low magnetic field levels, no mechanism for such an effect has been rigorously tested. While it is true that certain epidemiological studies suggest that low frequency (50 or 60 Hz) magnetic field exposure correlates with the enhanced incidence of cancers (including leukemia, breast and brain cancer), these same types of fields have not shown consistent association with genetic damage. This consideration suggests that a MF may act as a promoter or co-promoter, rather than an initiator, during carcinogenesis. Representative of this hypothesis is the skin cancer study using SENCAR mice which suggests that a MF can exert a co-promotional effect with the promoter 12-O-tetradecanoylphorbol-13-acetate (Stuchly *et al.*, 1992).

Studies relating extremely low frequency (<300Hz) magnetic fields to genotoxicity have included at least six positive and eighteen negative studies (McCann *et al.*, 1993). Such fields are Ames test negative; after exposure of Salmonella strains TA100 and TA98 to a magnetic field (100 Hz; 0.00013, 0.0013, 0.013, or 0.13 mT) for 48 or 6.5 hours respectively, no increases in revertant numbers were observed (Juutilainen & Limatainen, 1986).

Using a 50 Hz 5 mT MF, Fairbairn & O'Neill assayed for pulsed MF mediated DNA strand breakage using the single cell gel (SCG, or 'comet') assay on several immortalized human cancer cell lines. Neither the MF alone nor the MF in the presence of hydrogen peroxide were capable of damaging DNA (Faribairn & O'Neill, 1994). Conversely, using a similar technique Lai & Singh (1997) have shown dose dependendent induction of single stranded DNA breakage after exposure to 60 Hz MFs of 0.1-0.5 mT, and of double strand breakage at 0.25 and 0.5 mT. Discussing the disagreement between these two experiments, Lai & Singh argued that Fairbairn's method (where cells suspended in agarose were exposed to the magnetic field) did not provide a sound physiological environment for the cells during MF exposure and would thus prevent MF influences on enzymatic processes. It is possible, however, that the disagreement stems from the differences in the MF employed in the two experiments (pulsed versus continuous), or may reflect differences in response between the cell types assayed (human HL-60, Raji, and HeLa cancer cells versus rat brain cells).

Using a 50 Hz 400 mT MF, Miyakoshi *et al.*, (1995) have demonstrated enhancement of *hprt* mutant frequency by the MF, both with and without X-ray exposure, in human

malignant melanoma (MeWo) cells. The mutant frequency enhancement (a six-fold increase was observed after a 20 hour exposure) was shown to be dependent on the duration of MF exposure and on the induced current intensity (Miyakoshi *et al.*, 1995). Since MF's had not been shown to be capable of inducing DNA damage and stand breaks previous to their study, and since the mutant frequency enhancements disappeared when an inhibitor of DNA replication was included, the authors concluded that the observed increases may reflect an increase in DNA replication errors caused by the MF. Koana *et al.* (1997) have suggested that Miyakoshi's results could instead be explained by a MF induced stabilization of radicals spontaneously produced in the MeWo cells.

Further indirect evidence for the radical stabilization hypothesis stems from the study of wing spot mutants in *Drosophila melanogaster* by Koana et al. (1997). They observed more than a doubling of wing spot mutants in flies exposed to a 5 T MF over unexposed controls ($p<0.01$). Since this type of mutation is thought to be caused by somatic recombination, and since vitamin E (an antioxidant) suppressed the MF effect, the authors concluded that their results were likely the result of radical stabilization.

Most relevant to the motivation of the present work is the study by Rosenthal and Obe (1989) wherein human peripheral lymphocytes (HPL) were pretreated with MNU or trenimon and then exposed to 50 Hz magnetic fields ranging from 0.1-7.5 mT. Statistically significant ($p<0.05$) increases in sister-chromatid exchanges (SCE) per cell were reported for cells exposed to a 7.5 mT field and pre-exposed to either 0.5 or 1 mM MNU. Further, dose dependent increases in SCE were found in HPL's exposed to a MF ranging from 0.1 to 7.5 mT and pre-exposed to 1×10^{-7} M of the quinoid

drug trenimon. Since in all cases the SCE increase was less than a doubling of the SCE background frequencies, the authors concluded that their results could be explained by changes in composition of HPL subsets rather than from genetic damage (Rosenthal & Obe, 1989). It is also possible, however, that the increase in SCE's in cells exposed to trenimon resulted from stabilization of semiquinone radicals produced by reduction of the drug within the lymphocytes. This explanation is also supported by the nature of the dose response (increasing damage with increasing MF strength) since MF induced stabilization effects on random radical-radical reactions also increase with field strength (Cozens & Scaiano, 1993).

1.5 Radical Stabilization

The notion of MF influence on free radical reactions has been discussed as early as 1969 by Brocklehurst, who proposed that if a magnetic field could change electron relaxation times then it could effect the ratio of singlet and triplet recombination channels of radical-ion pairs (Molin, 1984). The phenomenon is established in the physico-chemical literature (Molin, 1984; Okazaki, *et al.*, 1987; Cozens & Scaiano, 1993; Scaiano, 1995a; 1995b; 1995c), and involves the perturbation of the spin evolution of triplet radical pairs - thus inhibiting the reformation of the singlet state in favour of the more reactive triplet state, as described below. Recent work suggests that both DNA and protein radicals are subject to MF-mediated stabilization (Boch *et al.*, 1996, Scaiano, 1995a; Mohtat *et al.*, submitted).

A radical pair derived from a parent molecule will exist either in the singlet (opposed electron spin) or triplet (same spin) state. A magnetic field of sufficient intensity causes Zeeman splitting of the triplet sublevels (T_+, T_0, T_-) in a triplet radical pair (Scaiano, 1994), thus inhibiting the intersystem crossing of the triplet to the singlet state (see Fig.1.1). Since reaction to ground state (parent) products occurs from a singlet radical pair, the MF increases the fraction of triplet pairs, and hence increases the fraction that succeed in separating (Scaiaino, 1994). If the radical yield is magnetic field dependent, then for certain chemicals one expects more radicals available for DNA insult. Depending on the reactants (a hydrogen acceptor and a hydrogen donor) the static field at which 50% of the maximum effect can be achieved ($B_{1/2}$) ranges from about three to several hundred mT (Scaiano, 1995a). In the laser flash photolysis

experiments conducted by Scaiano *et al.*, the MF stabilized reactions occurred within micelles (Cozens & Scaiano, 1993; Scaiano, 1994). There is reason to believe that in real cells the $B_{1/2}$ for a given reaction would be significantly lower than that found in the smaller micelles since the general observation in these experiments is that larger aggregates yield stronger MF influences (Scaiano, 1995a).

It is known that oscillating fields of low frequency (50-60 Hz) produce similar effects as the static fields used in the abovementioned studies due to the extreme rapidity of radical pair processes (which occur on a much shorter time scale than 60 Hz; Scaiano, *et al.*, 1995c; Scaiano *et al.*, 1994).

$$R\text{-}R \Leftrightarrow R^{\bullet\uparrow} + R^{\bullet\downarrow} \qquad \text{SINGLET}$$

$$\Downarrow \text{(Magnetic field prolongs triplet state)} \Downarrow$$

$$R\text{-}R \Rightarrow R^{\bullet\uparrow} + R^{\bullet\uparrow} \qquad \text{TRIPLET}$$

Figure 1.1 Magnetic Field Mediated Radical Stabilization

A radical pair may exist in either the singlet (top) or triplet (bottom) state. It is expected that 75% of random radical encounters will be triplet pairs (Cozens & Scaiano, 1993). An applied magnetic field inhibits triplet to singlet state changes.

1.6 The Chemicals

Menadione (vitamin K_3, 2-methyl-1,4-napthoquinone) is an antineoplastic agent representative of many quinoid drugs used in chemotherapy, and is undergoing trials for the treatment of gastrointestinal cancers (Tetef *et al.*, 1995). Menadione is a lipid soluble redox cycling quinone which is known to induce single and double- stranded DNA breaks in human cells. The cytotoxicity of menadione in human breast carcinoma (MCF-7) cells is inhibited by catalase, implicating the hydroxyl radical as a conduit for menadione induced damage (Nutter et al., 1992). Hydrogen peroxide production is involved in menadione induced DNA damage, as evidenced by the reduction of such damage in MCF-7 cells by α-ketoacids (Nath, *et al.*, 1995).

There are at least two pathways of metabolism of menadione in mammalian cells. In MCF-7 cells, Nutter *et al.* proposed that menadione (MD) undergoes a two electron reduction to dihydromenadione (MDH_2) catalyzed by NADPH quinone acceptor oxoreductase (QAO, also known as DT-diaphorase):

$$MD \xleftrightarrow[QAO]{2e} MDH_2$$

$$MDH_2 + O_2 \rightarrow MDH + O_2^{\cdot -}$$

$$MDH + O_2 \rightarrow MD + O_2^{\cdot -}$$

$$O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2$$

$$Fe^{3+} + O_2^{\cdot -} \rightarrow Fe^{2+} + O_2$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^-$$

It is unlikely that the 2-electron reduction pathway is a major pathway towards menadione induced mutagenesis since the resultant hydroquinones formed are relatively stable (Thor *et al.*, 1984; Sawada *et al.*, 1991).

It is also known that menadione is triggered by enzymes such as NAD(P)H-cytochrome P-450 reductase to experience a 1-electron reduction to the menasemiquinone radical, which in turn quickly oxidizes to regenerate menadione (thus forming the superoxide anion radical $O_2^{\bullet-}$) and thus initiates an intracellular superoxide radical burst (Sawada *et al.*, 1991). That the genotoxic potential of menadione is largely mediated by the 1-electron reduction pathway is evidenced by the dramatic reduction in NAD(P)H-cytochrome P-450 levels in menadione resistant Chinese Hamster Lung cells, which have a three-fold resistance to menadione induced chromosomal aberrations (Sawada, *et al.*, 1991).

Menadione was chosen as a test chemical for the radical stabilization hypothesis in this system for several reasons. First, it is mutagenic (Ono, *et al.*, 1995). Second, the susceptibility of photo-reduced menadione to MF induced radical stabilization has been demonstrated (Okazaki, *et al.*, 1988). Third, there are several possible ways that the menasemiquinone radical could become part of a radical pair subject to magnetic field effects - either by reaction of two independently generated menasemiquinone radicals, or by reaction of one menasemiquinone radical with a foreign radical species, or by the formation of radical pair intermediates between reduced menadione and any of several cellular hydrogen donors, including NADPH-cytochrome P-450 reductase, NADH-cytochrome b_5 reductase, and NADH-ubiquinone oxireductase (Thor *et al.*,

1982; Sawada *et al.*, 1991). If in fact radical pair intermediates are not formed during the 1-electron reduction pathway of menadione, we might still expect a magnetic field mediated effect for reasons described by Cozens & Scaiano in their study of MF influences on random radical reactions:

> "Whatever radical-molecule processes may take place in biological systems, the radical concentrations will be ultimately regulated by radical-radical processes which remove free radicals from the system. Our results show that such radical processes should be the subject of magnetic field effects, and that small fields are sufficient to cause significant effects." (Cozens & Scaiano, 1993)

The most likely route of MF effects on menadione chemistry, therefore, would involve the interaction of radical pairs that do not arise by decomposition of molecule-precursors, specifically involving the semiquinone radical with a randomly encountered radical. The electron spins of such 'uncorrelated' radical pairs are randomly oriented; ¼ are expected to be in the singlet state and ¾ in the triplet state (Molin, 1984). Note that random encounters between oxygen centred radicals are may have a more even distribution between the singlet and triplet states (approximately 1:1; Dr. C. Bohne, personal communication).

Both pathways of menadione metabolism lead to reactive oxygen species capable of damaging DNA. The superoxide anion radical and hydrogen peroxide take part in metal catalyzed reactions to form the hydroxyl radical and singlet oxygen (Thor *et al.*, 1982). DNA damage from the hydroxyl radical is only expected to occur when the radical is generated close to the DNA since the half-life of the hydroxyl radical is extremely short (10^{-9} seconds; Tchou & Grollman, 1993). Hydroxyl radical attack on

DNA is thought to be facilitated by transition metal ions (iron and copper ions) associated with DNA which are able to catalyze the reduction of hydrogen peroxide to yield the hydroxyl radical in close proximity to the DNA (Duell *et al.*, 1995; Imlay & Linn, 1988; Ward *et al.*, 1987). Both the hydroxyl radical and singlet oxygen (with a half life of 10^{-6} seconds) are able to cause 8-oxoguanine lesions (8-oxoG) (reviewed by Tchou & Grollman, 1993). One might expect menadione to induce 8-oxoG : A mispairs (and subsequently G:C → T:A transversions) owing to the attack of singlet oxygen and the hydroxyl radical on guanine. In *E.coli* at least, this does not seem to be the main source of menadione induced mutations as evidenced by the lack of significant induction of Trp^+ revertants in a *mutY* DNA-glycosylase deficient strain of E.*coli* exposed to menadione. One would expect the DNA-glycosylase deficient strain to reveal any 8-oxoguanine adducts by its inability to remove adenine misincorporation opposite 8-oxoG (Urios *et al.*, 1995).

Since the superoxide radical burst produced by menadione is similar to that catalyzed by iron in the Haber-Weiss type reaction (wherein hydrogen peroxide is reduced to water and the hydroxyl radical) we might expect menadione induced mutagenesis to have similarities with the spectrum produced by Fe^{2+}. If the spectrum for Fe^{2+} mutagenesis in *E.coli* described by McBride *et al.* (1991) is accurate then we would expect menadione to induce predominantly G→C transversions (by the formation of 8-hydroxyguanosine and the subsequent fixing of a G:G mispairing), C→T transitions (either by deamination of cytosine to give deoxyuridine mispaired with A, or by the formation of 6-hydroxy-5,6-dihydrocytosine) and G→T transversions (either by 8-

oxoG formation as mentioned above or by depurination with A being a frequent mispairing at abasic sites; McBride, *et al.*, 1991). Support for a menadione induced G:G mispairing comes from the observation of a high frequency of G:C→ C:G transversions in a *mutM* strain of E.*coli* after menadione exposure; presumably indicating the inability of this strain to remove 8-hydroxyguanosine lesions (Ono *et al.*, 1994): the MutM glycosylase removes 8-hydroxyguanine, 2,6-diamino-5-hydroxy-5-formamidopyrimidine, 8-hydroxyadenine and 4,6-diamino-5-formamidopyrimidine lesions in DNA (Tchou *et al.*, 1991; Ono *et al.*, 1995). If the R2λLIZ cells are proficient in the removal of menadione induced damage, then we may expect the ensuing abasic sites to be followed by slippage caused mutations (Ono *et al.*, 1995), or to result in G → T transversions owing to adenine misincorporation.

Menadione is also expected to induce deletions and rearrangements since oxygen radicals can cleave the phosphodiester DNA backbone (McBride *et al.*, 1991; Imlay & Linn, 1988), leading to sugar fragmentation, base loss and strand breakage (Imlay & Linn, 1988).

N-methylnitrosourea (MNU: $H_2NCON(NO)CH_3$) is a methylating agent and a direct-acting carcinogen (Liu *et al.*, 1994). The mutagenic potential of MNU is thought to stem from O^6-methylguanine, N^7 -methylguanine, O^2-methylthymine and O^4-methylthymine lesions in DNA (Liu *et al.*, 1994). O^6-methylguanine preferentially mispairs with thymidine during DNA synthesis, accounting for the G:C→ A:T transitions characteristic of MNU (Richardson *et al.*, 1987). Further, MNU treated

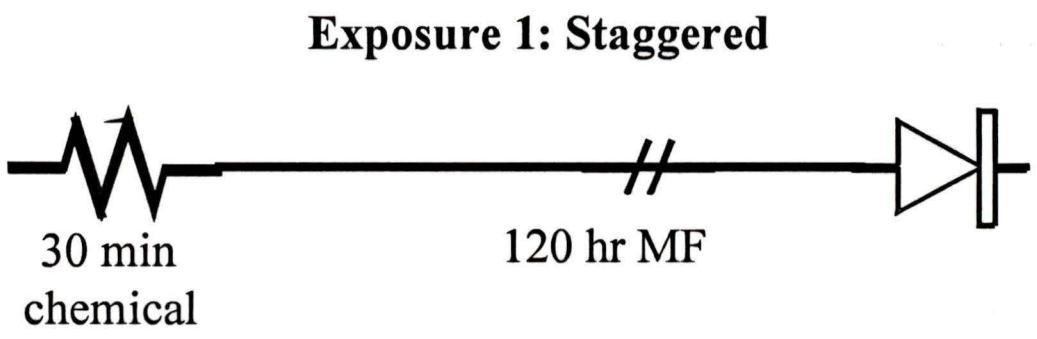
mice show G:C → A:T point mutations in K-ras codons 12 and 13 isolated from lymphomas (Mangues *et al.*, 1994).

Trenimon (2,3,5-triethyleneimino-1,4-benzoquinone) is an aziridinylbenzoquinone anticancer drug capable of alkylating -SH, -OH, and $-NH_2$ functional groups by opening of the ethyleneimine (also called aziridine) rings (Obe and Beek, 1979). Like menadione, trenimon may experience either two or one electron reductions by DT-diaphorase or NADPH cytochrome P450 reductase, respectively (Silva, *et al.*, 1992). Unlike menadione, however, the two electron reduction pathway of trenimon is probably a major mutagenic pathway since the resultant hydroquinone can experience opening of the aziridine rings and can subsequently alkylate macromolecules (Silva *et al.*, 1992). In the presence of oxygen, the hydroquinone can also be oxidized to a semiquinone radical, resulting in oxidative stress. Further, trenimon itself may be reduced by NAD(P)H-cytochrome P-450 reductase to experience a 1-electron reduction to the semiquinone radical, which in turn quickly oxidizes to regenerate trenimon (thus forming the superoxide anion radical $O_2^{\bullet-}$), initiating an intracellular superoxide radical burst (Silva *et al.*, 1992). Therefore, following the argument for menadione, the mutagenicity of trenimon might involve 8-oxoG : A mispairs owing to hydroxyl radical attack on guanine, and subsequently G:C → T:A transversion, and may be influenced by a MF acting on the abovementioned radical species. The alkylating potential of trenimon, however, is not expected to be directly enhanced by an applied MF.

1.7 Exposure Protocols

The mutagens were chosen for their known mutagenic ability and suspected mechanisms of mutagenesis. Menadione and trenimon are test chemicals in that they have the potential of damaging DNA via the formation of free radicals (ultimately superoxides and hydroxyl radicals). As an alkylating agent MNU is not thought to incur mutations through any radical mechanism, and so was chosen as the control chemical. Since the magnitude of any MF mutagenic enhancement is unknown, several chemical concentrations were tested in order to optimize the sensitivity of the experiments.

In terms of chemical-field co-exposure, two protocols were used. In the first (the staggered exposure), cells were pre-exposed to the chemical for 30 minutes, washed, and then put into the MF exposure apparatus after the design of Obe and Rosenthal (1989). Here the cells are "co-exposed" only in terms of the field and the chemical which has remained in the cell after the washing procedure. In the second (concurrent) exposure protocol the 30 minute chemical treatment coincided with a 30 minute MF exposure, whereafter the cells were washed and put back into the field (see Fig.1.2). Both protocols subsequently allow 120 hours of growth to confluency within the MF, allowing time for the fixation of mutation. By comparing the results obtained from these protocols, one may discern between an MF enhancement of mutagenesis by a mechanism such as radical stabilization and the confounding possibility of a MF enhancement of cell-membrane traversal of the mutagens.



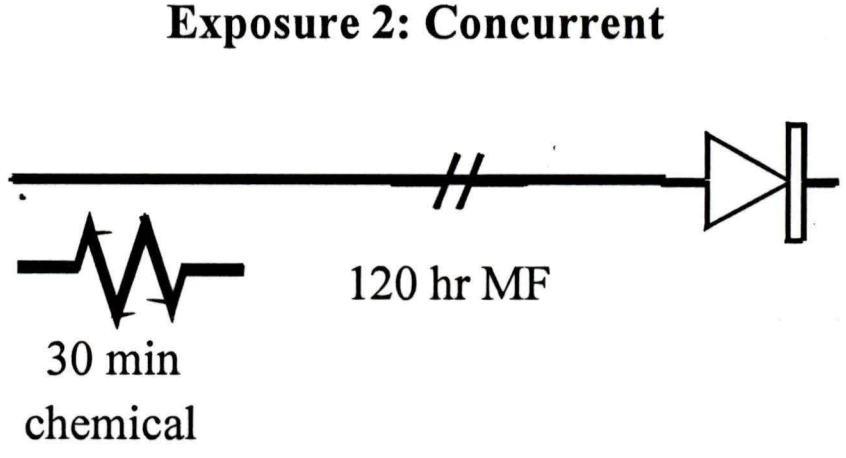


Figure 1.2: Exposure Protocols

1.8 Description of the Big Blue Rat Cell Line Protocol

The shuttle vector depicted in Fig. 1.3 is found in several tandem copies on two chromosomes in the R2λLIZ cell, totalling 50-70 copies per cell (Wyborski *et al.*, 1995). Karyotype analysis by fluorescent in situ hybridization led Wyborski *et al.* to conclude that R2λLIZ is a polyploid cell line with an average of 76 chromosomes per cell (2n=42 in the rat), and that since the two chromosomes which received the shuttle vector are similar in their morphology, integration sites, and copy number, it is likely that there was a chromosome duplication following a single integration event (Wyborski *et al.*, 1995). To allow generation of enough cells for experimentation, all exposure groups in the present study were exposed on the fifth passage of the cells from the primary stock. This number of passages was chosen since Wyborski *et al.* found no changes in either the number of integration sites or the chromosome number after five passages (Wyborski *et al.*, 1995).

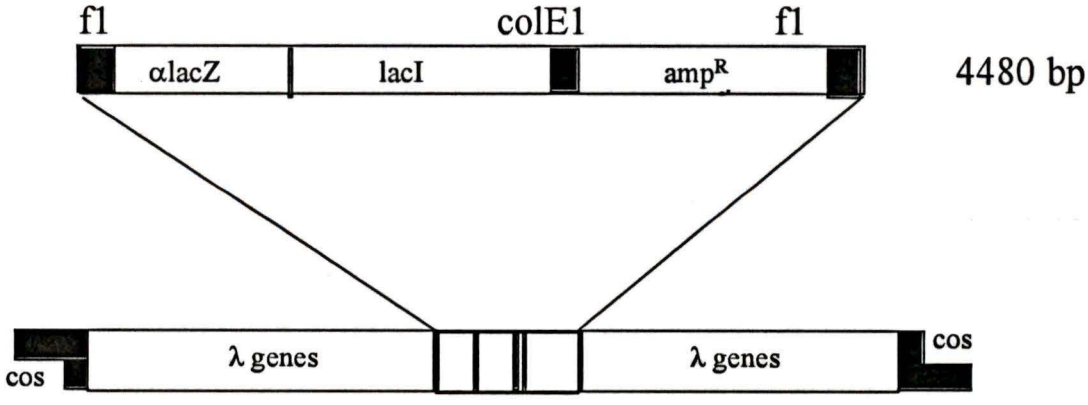


Figure 1.3: The R2λLIZ Shuttle Vector

The top bar in Figure 1.3 represents the phagemid (4480 bp) embedded in the lambda DNA (45.5 kb). The entire construct is packaged into bacteriophage lambda particles though by *in vitro* packaging extract, after which the particles are screened for mutant *lacI* genes within *E.coli*. α*lacZ* (750 bp) is transcribed within the host bacteria (SCS-8) and compliments the carboxyl portion of the *lacZ* gene within the host.

The protocol is designed to detect mutations in the *lacI* gene of this construct. Following exposure, the *lacI* transgene is rescued by λ packaging extract (the phage head particles and proteins required for packaging of the concatamers into phage heads). Packaged phage are used to infect a *lacI*$^-$ strain of *E.coli* (SCS-8). Between the phage and the bacterium a functional *lacZ* product, β-galactosidase, can be made by α-complementation in the absence of a functional repressor of the *lac* operon, the *lacI* gene. Thus, those phage which packaged functional *lacI* genes repress β-galactosidase production within SCS-8. Mutant *lacI* is detected and isolated by including the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) in the NZY medium: they are blue owing to the cleavage of X-gal to a blue product by β-galactosidase.

1.9 Lac I Molecular Analysis

The *lacI* gene is a 1,080 base pair region of the *E.coli* chromosome which codes for the lac repressor protein of 360 amino acids (Farabaugh, 1978). The detection of mutations within this gene is made possible by the polymerase chain reaction (PCR) of the target DNA, yielding a 1254 bp fragment, and by conducting chain terminating sequencing reactions on the PCR products. The DNA sequence is determined with the use of an automated laser fluorescence (ALF) sequencer. Previous study of the *lacI* transgene has shown 365/1087 mutable sites (sites in the *lacI* gene which have the potential of revealing a mutation in a bacterial detection system), and a higher proportion (113/177) in the nc+ (DNA binding site of the repressor protein) coding region of the gene (deBoer, personal communication).

The *lacI* gene also contains 95 CpG sequences (Farabaugh, 1978), which are thought to contribute significantly to the occurrence of G:C→A:T transitions in the Big Blue® systems owing to the production of 5-methylcytosine residues (within the eukaryotic cells) and their subsequent deamination to thymine (Kohler et al., 1991b).

CHAPTER II - METHODS

2.1 Methylene Blue Viability Staining

Each of three round tissue culture plates were seeded with 500 cells (as determined by hemocytometer counting and dilution) and exposed to 0, 1, 2, 5 or 10 μg/ml menadione (final concentration in 10 ml of media) for 30 minutes. MNU exposure groups were seeded at 200 (no MNU), 300 (75 μg/ml), 500 (100 and 150 μg/ml) or 1000 (200 μg/ml) cells, and exposed to ethanolic MNU for 30 minutes. Trenimon groups were seeded with 385 cells and exposed to 0, 0.5, 1, 2 or 5 x 10^{-8} M of ethanolic trenimon. Colonies from surviving cells were large enough for the methylene blue fix-stain procedure after 8 days of incubation (37°C , 7% CO_2). At this time the media was removed, cells were washed once with Phosphate Buffered Saline (PBS), and 5 ml of fix-stain solution (1% methylene blue, 50% methanol) was added to each plate. After 45 minutes the solution was removed and the plates washed twice with water. Plates were air dried overnight and blue colonies counted. Data are shown in Table 3.1 and Figure 3.1.

2.2 R2λLIZ Cell Culture

The rat embryo fibroblast cell line (obtained from Stratagene, LaJolla, CA) R2λLIZ was grown in Dulbecco's Modified Eagle Medium (DMEM; Gibco BRL, Gaithersburg, MD) supplemented with 10% Fetal Bovine Serum (FBS; Gibco), 200 μg/ml geneticin (Sigma, St. Louis MO) and 10 μg /ml gentamycin for a total volume of 10 ml per tissue culture flask. Three 25 cm^2 polystyrene flasks (Corning, New York N.Y.) were used per data point. After a preincubation period of 24 hours the fibroblasts were exposed to the appropriate concentration of chemical in ethanol for 30 minutes. Controls with and without ethanol were also included.

Temperature stability was monitored by periodical thermometer measurement in both double distilled H_2O and complete media inside and outside the magnetic field coils. A CO_2 level of 7% was confirmed periodically.

2.3 R2λLIZ Chemical and Magnetic Field Exposures

A thermostable MF generator was designed and built by the Department of Electrical Engineering at the University of Victoria to deliver a uniform flux density. The exposure magnetic field was produced by coils in the Merritt configuration (Merritt, 1983). The system consisted of 4 rectangular coils which housed a volume of 7000 cm^3. The windings of the coils were electrostatically shielded, eliminating the electric field. The magnetic field in the volume occupied by the cells was uniform within at least 5 %, as computed and confirmed experimentally (Caputa and Stuchly,1996). Coil vibration was eliminated by epoxy encapsulation of the coils. The cell preparations were placed on a support separated from the coil system in a radially symetrical configuration relative to the exposure coils. The exposure magnetic field of 3 mT at 60 Hz was produced by current supplied to the coils through a variac from a standard 115V/15A power outlet. The field strength was monitored daily with a Metex® Digital Multimeter (Electric Field Measurements, MA) to ensure a constant flux density of 3 mT within the coils of the generator. Background Fields in the control incubator were 2×10^{-4} mT. All exposure groups were also subject to the earth's static magnetic field (approximately 50 μT).

In the staggered exposure protocol, cells of the fifth passage were grown to 17 ± 2 % confluence [$(2.10 \pm 0.02) \times 10^5$ cells/ml], exposed to several concentrations of menadione, trenimon, or MNU for 30 minutes, washed with PBS, and then given fresh growth media. Each exposure group was pooled from 3 separate flasks, each containing 10 ml of cells. At the time of chemical exposure, then, each exposure

group contained (6.30 ± 0.06) x 10^6 cells. Cells were grown for 120 hrs to 100% confluency [(1.24±0.11) x 10^6 cells/ml] either within the MF coils (3mT) or in the control incubator (2 x 10^{-4} mT). Therefore after exposure and growth to confluence an exposure group contained approximately (37 ± 3.3) x 10^6 cells.

In the concurrent exposure protocol, cells of the fifth passage were grown to 17 ± 2 % confluence, exposed to several concentrations of menadione, trenimon, or MNU (and the exposure groups were exposed to a 3 mT magnetic field during this 30 minute chemical exposure), washed with PBS, given fresh growth media and returned to either the MF coils or to the control incubator until confluency (120 hrs).

After exposure and growth to confluence cells were trypsinized (2 ml 0.25% trypsin for 30 seconds) resuspended in 10 ml PBS, and cells from the three flasks were pooled into one 50 ml polypropylene tube and centrifuged for 10 minutes at 2500 g. The cell pellet was then resuspended in 1 ml PBS in a 1.5 ml cryotube, recentrifuged in a microcentrifuge (2500 g, 10 minutes), the supernatant removed, and the pellet stored at -80 °C to await DNA isolation.

2.4 Extraction of Genomic DNA by Dialysis

DNA was isolated using the dialysis method (Winegar, unpublished; Stanford Research Institute, Menlo Park CA). Cells were thawed and suspended in 5 ml Cell Lysis Solution (CLS; 10 mM TrisHCl, pH 8.3, 140 mM NaCl, 3 mM KCl, 0.35 M sucrose, 1 mM EDTA, 1% Triton X-100) in a douncer (on ice). The pellet was dounced ten times with pestle B and then ten times with pestle A (on ice). Dounced cells were strained through a 100 micron nylon mesh filter into a 50 ml polypropylene tube, and spun 12 minutes at 1100 g at 4 °C. The tube was swabbed with a Q-tip, and the pellet rinsed with 3 ml CLS, followed by recentrifugation at the above conditions for 5 minutes. After swabbing, 55 µl of Dounce Buffer (phosphate buffered saline with EDTA; 12.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 13.7 mM NaCl, 2.7 mM KCl, 10 mM EDTA, pH 8.0, with 20 µl/ml RNAce It) was added to remove the pellet from the wall of the tube. Then 55 µl of prewarmed (50 °C) proteinase K/SDS solution (0.1 mg proteinase K, 100 µl 10 % SDS, 100 µl 0.5 M EDTA pH 7.5, 300 µl ddH_2O) was added, the solution was gently stirred, and incubated for 15-20 minutes at 50 °C. Following incubation, the digestion was transferred, with a wide bore pipette, to a 0.45 µm filter (dia. 30 mm, Millipore's "Cell Culture Plate Insert") floating in 35 ml of TE buffer. After 18-48 hours of dialysis the genomic DNA was transferred to a 1.5 ml minitube (Eppindorf) with a wide bore micropipette tip to prevent shearing of the DNA.

2.5 Packaging of the lac I λ Phage Shuttle Vector

Packaging reactions were done by mixing 8 µl of genomic R2λLIZ DNA with 10 µl of Stratagene's Transpack packaging extract and incubating for 90 minutes at 30 °C. Following this incubation, another 10 µl Transpack was added and another incubation (90 minutes at 30 °C) followed. The packaged mixture was then diluted to 1 ml with SM buffer and stored at 4°C with 50 µl $CHCl_3$. Packaging reactions were carried out in sets of six; packaging reactions were coded (for example as sample "A1") prior to assaying for mutant frequencies (see Table 2.1).

Table 2.1 Code of Packaging Reactions

Sample	Packaging Reaction Designation						
	1	2	3	4	5	6	7
1	I_6C_s	I_6C_s	I_2C_s	I_2C_s	I_2C_s	I_2C_c	I_2M1_c
2	I_6C_s	I_6M1_s	I_6C_s	I_6C_s	I_6C_s	I_8C_c	I_8M1_c
3	$I_2M2.5_s$	$I_2M2.5_s$	I_2M1_s	I_2M1_s	I_2M1_s	I_2E_c	$I_2M2.5_c$
4	$I_6M2.5_s$	$I_6M2.5_s$	I_6M1_s	I_6U20_s	$I_2M2.5_s$	I_8E_c	$I_8M2.5_c$
5	I_2M5_s	I_2M5_s	I_2U20_s	I_2U100_s	I_2U100_s	I_2M1_c	I_2M5_c
6	I_6M5_s	I_6M5_s	I_6M5_s	I_6U100_s	I_6U100_s	I_8M1_c	I_8M5_c
	Packaging Reaction Designation						
	8	9	A	B	C	D	E
1	I_8C_c	I_2C_c	I_8C_c	I_8C_c	I_2C_c	I_2C_c	I_2M1_c
2	I_2M1_c	I_8C_c	I_8C_c	I_8C_c	I_8C_c	I_8C_c	I_8M1_c
3	I_8M1_c	I_2E_c	$I_2M2.5_c$	I_2U20_c	$I_2M2.5_c$	$I_2M2.5_c$	$I_8M2.5_c$
4	$I_8M2.5_c$	I_8E_c	$I_8M2.5_c$	I_8U20_c	$I_8M2.5_c$	$I_2M2.5_c$	I_2M5_c
5	I_2U20_c	$I_2M2.5_c$	I_2U100_c	I_2U100_c	I_8U20_c	I_2U100_c	I_8M5_c
6	I_8U20_c	I_8M5_c	I_8U100_c	I_8U100_c	I_2U100_c	I_2U100_c	I_8U100_c
	Packaging Reaction Designation						
	F	G	H	I	J	-	-
1	$I_8M2.5_c$	$I_2C_{s/c}$	$I_8C_{s/c}$	$I_2T7.5_{s/c}$	$I_8T7.5_s$	-	-
2	$I_8M2.5_c$	$I_8C_{s/c}$	$I_2T0.5_{s/c}$	$I_8T7.5_s$	$I_8T7.5_s$	-	-
3	I_8M5_c	$I_2U100_{s/c}$	$I_8T0.5_s$	$I_8T7.5_s$	I_8U100_s	-	-
4	I_8M5_c	$I_2T5_{s/c}$	$I_2T7.5_{s/c}$	I_8U100_s	I_8U100_c	-	-
5	I_2U20_c	$I_8T0.5_c$	$I_8T7.5_s$	I_8U100_c	$I_2C_{s/c}$	-	-
6	I_2M5_c	$I_8T0.5_s$	$I_8T7.5_c$	$I_8T7.5_c$	$I_8T0.5_c$	-	-

Key: I_2, control incubator (2×10^{-4} mT); I_8 & I_6, MF incubator (3 mT); C, control (no chemical); E, (10 ul/ml 100% EtOH), MX (X μg/ml menadione); TX (X x 10^{-9}M trenimon); UX (x μg/ml MNU); $subscript_s$, staggered exposure; $subscript_c$, concurrent exposure; $subscript_{s/c}$, equivalent exposure (since 'staggered' and 'concurrent' only have meaning when both field and chemical are applied).

2.6 Mutant *lac I* Selection and Purification

Putative mutants were selected following transfection of the *lacI* deficient E.*coli* strain SCS-8 and plating on 25 x 25 cm plates (Stratagene) containing top agarose with 1.5 mg/ml X-gal as detailed by Kohler *et al.*, (1990; 1991a; 1991b), and Rogers *et al.* (1995).

In the titre step, 2 ml of SCS-8 was infected with 50 µl of the packaged phage mixture for 15 minutes at 37°C, mixed with 35 ml top agarose, and plated on 25 cm x 25 cm plates containing 250 ml of bottom agar (NZY). This was done in duplicate for each packaging reaction. The remainder of the packaged phage (approximately 900 µl per packaging reaction) was stored at 4°C overnight. After a 16-18 hour incubation at 37°C, plaques produced by lytic λ phage bursts were visible on the plates (most of which were clear in the titre step though the occasional blue plaque was isolated). An estimate of total plaque forming units (pfu) per packaging reaction was calculated by averaging the plaque counts from these titre plates and correcting for the 1/20 dilution. Titre counts were included in the calculation of the total number of pfu's for a given packaging reaction. An optimum of 11500 pfu/plate was used in all cases to estimate the number of 25 x 25 cm plates needed for the rest of the packaging reaction.

The remainder of the packaged phage was used to infect SCS-8 (2 ml/plate) for 15 minutes at 37°C as in the titre step. Following plate incubation at 37°C for 16-18 hours the total pfu was calculated by counting the number of plaques on 1/20 of the plate area on at least 3 plates: the average of these counts (± standard deviation) multiplied by the total number of plates plus the counts from the titre plates yielded the

total number of pfu per packaging reaction. Blue plaques were identified (using a red filter and a standard light table), picked from the plate using a pasteur pipette, and resuspended in 500 µl SM buffer with 50 µl of $CHCl_3$. Control mutant colour standards (CM_0 and CM_1) were plated alongside each set of six packaging reactions.

All putative mutants were verified and purified by replating. Purified blue plaques were cored with a sterile pasteur pipet and transferred to a screw cap microcentrifuge tube containing 500 µl SM to which a drop of chloroform was added.

Mutant frequency was calculated as the number of blue plaques divided by the total number of plaques plated (including the titre counts) ± standard deviation. The mutant frequencies presented in Tables 3.2 and 3.3 describe a range derived from the inclusion of the standard deviation (calculated from the method of counting total plaque numbers) in the mutant frequency calculations.

2.7 PCR of Mutant *lac I* Genes

PCR involved mixing 1 µl of purified mutant phage stock as the template in a final volume of 50 µl PCR amplification reaction [5 µl 10X PCR buffer (150 mM Tris-HCL pH 7.8, 15 mM $MgCl_2$, 600 mM KCL), 200 µM dNTP's, 10 pmol P2 forward primer, 10 pmol P9 reverse primer, and 5 µl Taq DNA polymerase]. Each of the 30 cycles of PCR programmed into a Perkin Elmer 9600 thermocycler consisted of denaturing, annealing and extension phases: 95 °C for 36 seconds, 51 °C for 36 seconds, followed by 72 °C for 90 seconds. PCR product quality was then checked on an agarose minigel. Primer sequences used to amplify the 1254 bp fragment containing the *lacI* gene in the PCR were:

P_2 (forward) positions -53 to -37 (5'-CCCGACACCATCGAATG-3'), and

P_9 (reverse) and positions 1201 to 1185 (5'-ACAATTCCACACAACATAC-3').

The PCR product was purified using a Promega Magic PCR prep DNA purification kit. Twenty min-prep columns were attached to a vacuum manifold and to a 3 ml syringe. 1 ml of PCR prep resin was mixed with each PCR sample and loaded into the syringe. A Barnant vacuum pump was used to draw this solution through the columns. 2 ml of 80% isopropanol was applied to wash the columns, which were then dryed by again applying the vacuum for 2 minutes. The columns were then centrifuged and transferred to a sterile epindorf tube. The DNA was eluted by the addition of 50 µl of ddH_2O to the column followed by microcentrifugation to pool the DNA solution.

2.8 Sequencing of Mutant *lacI* Genes

Sequencing runs were performed in blocks of 10 mutants on an automated laser fluorescence DNA sequencer (ALF; Pharmacia).

Sequencing reactions were performed in sets of 20 mutants. 2 µl of purified PCR template and 2 µl of the chosen sequencing primer were pipetted into tubes labelled 1-20 (one tube per mutant sample). 4 µl of A, C, G, or T termination mix (60 µM dNTP, 0.8 µM ddATP; 0.4 µM ddCTP; 0.08 µM ddGTP; or 0.8 µM ddTTP) was pipetted into the appropriate PCR tube. 18.5 µl of reaction mix [84 µl 10x sequencing buffer (300 mM Tris/HCl pH 9.0, 300 mM KCl, 50 mM $MgCl_2$), 31.5 µl primer, 260 µl water, and 21 µl TAQ polymerase] was added to each tube labelled 1-20. 5 µl from each of these was pipetted into the corresponding four PCR tubes. Cycle sequencing was performed in a Perkin Elmer 9600 thermocycler programmed to run 2 min at 94 °C, followed by 25 cycles of 10 seconds at 94 °C, 20 seconds at 50 °C, 30 seconds at 72 °C, followed by storage at 4 °C . After these cycles, 6 µl of stop dye (deionized formamide with dextran blue dye) was added to each tube. Prior to running the sequencing gel, samples were denatured for 2 minutes (95 °C) and immediatley placed on ice. 7.5 µl of sequencing reaction (A, C, G, or T) was loaded into each lane of the sequencing gel (A, C, G or T lanes).

Sequencing primers used included the forward primers:

G1: -41 to -20 5'-GAATGGTGCAAAACCTTTCGCG-3'

G2: 249 to 271 5'-ACGCGCCGTCGCAAATTGTCGCG-3'

G3: 532 to 549 5'-ACTGGGCGTGGAGCATCT-3'

G4: 816 to 833 5'-GCGTTGGTGCGGATATCT-3'

and the reverse primers:

H1: 305 to 282 5'-TGGCACCCAGTTGATCGGCGCGA-3'

H2: 573 to 550 5'-ATTTGCTGGTGACCCAATGCGACC-3'

H3: 848 to 825 5'-CGTATCCCACTACCGAGATATCCG-3'

H4: 1151 to 1128 5'-TGCCTAATGAGTGAGCTAACTCAC-3'

The primers are labelled with fluorescein at their 5' end.

Sequencing gels were prepared using Pharmacia Ready-Mix Gel-ALF Grade. The gel mix was brought to room temperature from storage at 4 °C, mixed with 480 µl of 10% ammonium persulfate, and poured between the assembled glass gel plates. Gels were allowed to polymerize for 3 hours (or overnight at 4 °C).

The detectors in the ALF recorded the passage of primer-tagged DNA fragments through the gel; as a fragment crossed the detector the laser generated by the ALF caused the fluorescein labeled primer to fluoresce, allowing the signal to be recorded into an ALF datafile which was converted to a chromatogram using Seqman (DNAstar) sequence analysis software.

2.9 Statistical Analyses of Mutant Frequencies

A Kastenbaum-Bowman analysis of the mutant frequency data from Tables 3.2 and 3.3 was conducted to test the hypotheses that the MF is neither mutagenic, co-mutagenic, nor antimutagenic. The mutant frequency data for menadione and MNU, including the Kastenbaum-Bowman analysis presented herein, has been published (Suri *et al.*, 1996). By employing the statistical approach of Kastenbaum and Bowman (1970), the exposure groups have been analyzed against the null hypothesis H_0: $\pi_1=\pi_2$, where π_1 is the mutant frequency within the field and π_2 is the frequency outside of the field. The calculations are based upon the smallest number of mutants, c, in *n* independent Bernouilli trials such that

$$\sum_{r=c}^{n} \binom{n}{r} p^r (1 - p)^{n-r} \leq \alpha$$

where p is the relative proportion of mutants in a single trial, and $0 \leq \alpha \leq 1$ is a specified level of significance. C is the "rejection number" which defines a critical region of size no larger than α. Following Kastenbaum's example, the hypothesis $\pi_1=\pi_2$, that the mutant frequency is the same for MF exposed and non-exposed groups, was tested against the alternative $\pi_1>\pi_2$, that the mutant frequency is higher in the exposed group (group 1). Having observed x_i mutants among a large number of target genes, N_i, derived from exposed R2λLIZ cells within the field (group 1) or

outside of the field (group 2), the above equation is adapted (where $n = x_1 + x_2$) such that if the preassigned level of significance is α, then we will reject H_0 if $x_1 \geq c$. The calculation works by the assumption that under H_0, the ratio $m = x_1 / (x_1 + x_2)$ may be treated as a binomial variable with conditional expectation:

$$E(m \mid x_1 + x_2) = N_1 / (N_1 + N_2)$$

and thus the relative proportion of mutants, p, between groups 1 and 2 under H_0 is $N_1 / (N_1 + N_2)$. The Kastenbaum-Bowman analysis of the data from Table 3.2 is presented in Table 3.5. A similar approach was used to test the hypothesis that the MF is not antimutagenic. Again the null hypothesis H_0 , states that $\pi_1=\pi_2$, where π_1 is the mutant frequency within the field and π_2 is the frequency outside of the field. The alternative hypothesis, however, is that $\pi_1<\pi_2$, that the mutant frequency is lower in the exposed group (group 1). Using similar assumptions, H_0 is rejected if $x_2 \geq c$. Note that in this case $p= N_2 / (N_2 + N_1)$. This Kastenbaum-Bowman analysis of the data from Table 3.2 is found in Table 3.6.

The same battery of analyses were performed on the concurrent data from Table 3.3 and the results are found in Tables 3.7 and 3.8. In certain cases (eg. MNU 100 µg/ml from the concurrent exposures) the available tables on the Kastenbaum-Bowman statistic were inadequate for the tabulations required by this study since values of 'c' have only been tabulated for $n < 200$. For some of the following comparisons the total number of mutants exceeds 200 and so a normal approximation to the binomial probability distribution was performed, as described by Byrkit (1987) and satisfying

the criterion of McPherson (1990) that $n_{min} > 2 \div \{p(1-p)\}^2$ in all cases. Here the variance, σ, is calculated as $\{np(1-p)\}^{1/2}$ and used to calculate the rejection number, c, from $Z = (X-M)/\sigma$ where X=c, M=np, and Z has been obtained from $P(x>c) = p - P(N)$, where P(N) is the area under the normal curve (Byrkit, 1987). Hence, where n is large: $c = Z\sigma + M$.

Note that a magnetic field exposed group is considered to have a significantly higher mutation frequency than the non-exposed group when $x_1 \geq c_{\alpha=0.05}$. Conversely, in the test for antimutagenicity within the field is significant when $x_2 \geq c_{\alpha=0.05}$. Tables 3.5 to 3.9 also show rejection numbers for $\alpha=0.01$.

Lastly, Table 3.9 shows the Kastenbaum-Bowman test for mutagenicity of menadione, MNU, and trenimon in the absence of the magnetic field. Here the test uses π_1 as the mutant frequency from the chemical exposure group and π_2 as that of the corresponding control group.

The mutagenicity and anti-mutagenicity of the MF and of the chemicals was also tested by Traut's method (Traut, 1980). This method has the advantage of yielding an exact *P* value for each significance test. Given a control mutant frequency *u* (ie. the mutant frequency from the no-field exposure group), and a test frequency of x_1/N_1 (where, as in the Kastenbaum-Bowman test, x_1 is the number of mutants in the MF-exposed group and N_1 is the number of plaques screened), the probability of finding $\geq x_1$ mutants in the exposed group (a test of mutagenicity), is given by Poisson's law such that:

$$P = \sum_{x_1}^{\infty} (m^x e^{-m})/(x!)$$

where $m = uN_1$ is the sample mean. Similarly, the probability of finding $\leq x_1$ mutants in the exposed group (a test of anti-mutagenicity), is found by summing from 0 to x_1 in the above equation (rather than from x_1 to ∞). Knowing x_1 and m, the P values were found using tables of the cumulative terms of the Poisson distribution (published by the Defense Systems Department of the General Electric Company, 1962). For each comparison made with the Kastenbaum-Bowman test, the corresponding P value from the Traut test is given in the last column of Tables 3.5 - 3.9. Note that a treatment is considered mutagenic when the $P(\geq x_1)$ value from the Traut test is ≤ 0.05 (the probability of finding x_1 or greater mutants in the exposed group), and the treatment is considered anti-mutagenic when the $P(\leq x_1)$ is ≤ 0.05.

2.10 Statistical Analyses of Mutational Spectra

Significant differences between mutational spectra were sought using the method of Adams and Skopek (1987). Here the null hypothesis is that the two mutational spectra under consideration (eg. spectra generated with and without MF exposure) were drawn from the same population. In order to compare spectra, mutations were listed by mutational class and the two spectra entered into a 2 x n table, where n is the number of classes for which there is at least one occurrence (or 'count') in the two input spectra (classes for which neither spectrum contains a count are not included in the table). For example, if a spectrum of 5 G →A transitions was compared to one with 3 G →A and 2 C→T transitions, then the table would be:

5 3
0 2

This method is based on an algorithm which generates a Monte Carlo estimation of the p value of the hypergeometric test. The input table is entered into a program ("HyperG", priovided by Dr. Neal Cariello) which generates a number of random tables that contain the same number of mutants in each row as the input spectra. In all comparisons, 2500 iterations were made, meaning that 2500 random tables were generated. The algorithm assigns an estimate of the hypergeometric probability to every table (including the input table). Under the null hypothesis, the p value is determined as the proportion of random tables that are as improbable or more improbable than the input table (Adams and Skopek, 1987).

The mutational spectra were also compared in an attempt to find significant differences in the proportion of mutations belonging to a single mutational class. A novel statistical approach for the comparison of mutational proportions was developed: In two samplings of mutations, n_1 and n_2, taken from mutant populations, N_1 and N_2, with observed proportions p_1 and p_2 belonging to a single mutational class (where $p=x/n$, and $x \geq 5$ is the number of mutations belonging to a given mutational class), it is possible to test the null hypothesis, H_0: $\pi_1 = \pi_2$, that the population proportions are equal. After Byrkit (1987), we find the sample value of the test statistic $z^* = (p_1 - p_2)/s_{\pi'}$ and reject H_0 in favour of H_1: $\pi_1 > \pi_2$ if $z^* > z_\alpha$. The denominator of the test statistic is the sample standard error of the pooled estimate of π, termed π', such that:

$\pi' = (x_1 + x_2)/ (n_1 + n_2)$, and

$s_{\pi'} = [\pi'(1-\pi') (1/n_1 + 1/n_2)]^{1/2}$

In cases where n>0.05N (the sample of sequenced mutants is greater than five percent of the population of isolated mutant plaques), then a finite population correction factor (FPCF) was used, replacing $1/n$ by $[N-n / n (N-1)]$ in the equation for $s_{\pi'}$ (Byrkit, 1987). Finally, a 95% confidence interval was calculated for each difference in population proportion (ie., p_1-p_2). The interval is given by:

$p_1 - p_2 \pm z_{\alpha/2} s_{p1-p2}$, where

$s_{p1-p2} = [p_1(1-p_1)/n_1 + p_2(1-p_2)/n_2]^{1/2}$.

Again, where n>0.05N, a FPCF of $[(N-n)/(N-1)]^{1/2}$ was used . Tests of the proportion of certain mutational classes between exposure groups are found in Table 3.14.

CHAPTER III - RESULTS

3.1 R2λLIZ Cell Survival - Methylene Blue Trials

In order to determine dose levels for each chemical which clearly damage the R2 cells yet allow approximately 50% or greater cell survival, methylene blue staining was performed. As shown in Table 3.1, since non-exposed cells have about 50% survival (in terms of colony forming units), standardized survival values were calculated by equating the non-exposed survival values to 100% within each chemical trial. Note that 6 plates were used in the trenimon trials in order to reduce the error associated with this survival test.

Table 3.1 Cell Survival as Determined by Methylene Blue Staining

Chemical and Concentration	# Cells seeded	# Colonies Counted	Survival (%)	Standardized Survival (% ± s.d.)
MNU (0 μg/ml)	200	95, 126, 133	59	100±17,
(75 μg/ml)	300	155, 108, 139	45	76±19
(100 μg/ml)	500	230, 165	40	67±16
(150 μg/ml)	500	101, 149	25	42±11
(200 μg/ml)	1000	210, 215	21	36±0.6
Menadione (0 μg/ml)	500	253, 269, 252	52	100±4
(1 μg/ml)	500	263, 232, 216	47	91±9
(2 μg/ml)	500	223, 242, 245	47	91±5
(5 μg/ml)	500	111, 138, 139	26	50±6
(10 μg/ml)	500	27, 26, 34	6	12±2
Trenimon (0)	385	180, 158, 185, 157, 147, 144	42	100 ± 1.7
(0.5×10^{-8} M)	385	103, 115, 126, 114, 102, 119	29	69 ± 0.9
(1×10^{-8} M)	385	28, 42, 48, 38, 47, 52	11	26 ± .07
(2×10^{-8} M)	385	11, 7, 5, 5, 1, 4	1	2 ± 0.2
(5×10^{-8} M)	385	0, 0, 0, 0, 0, 0	0	0

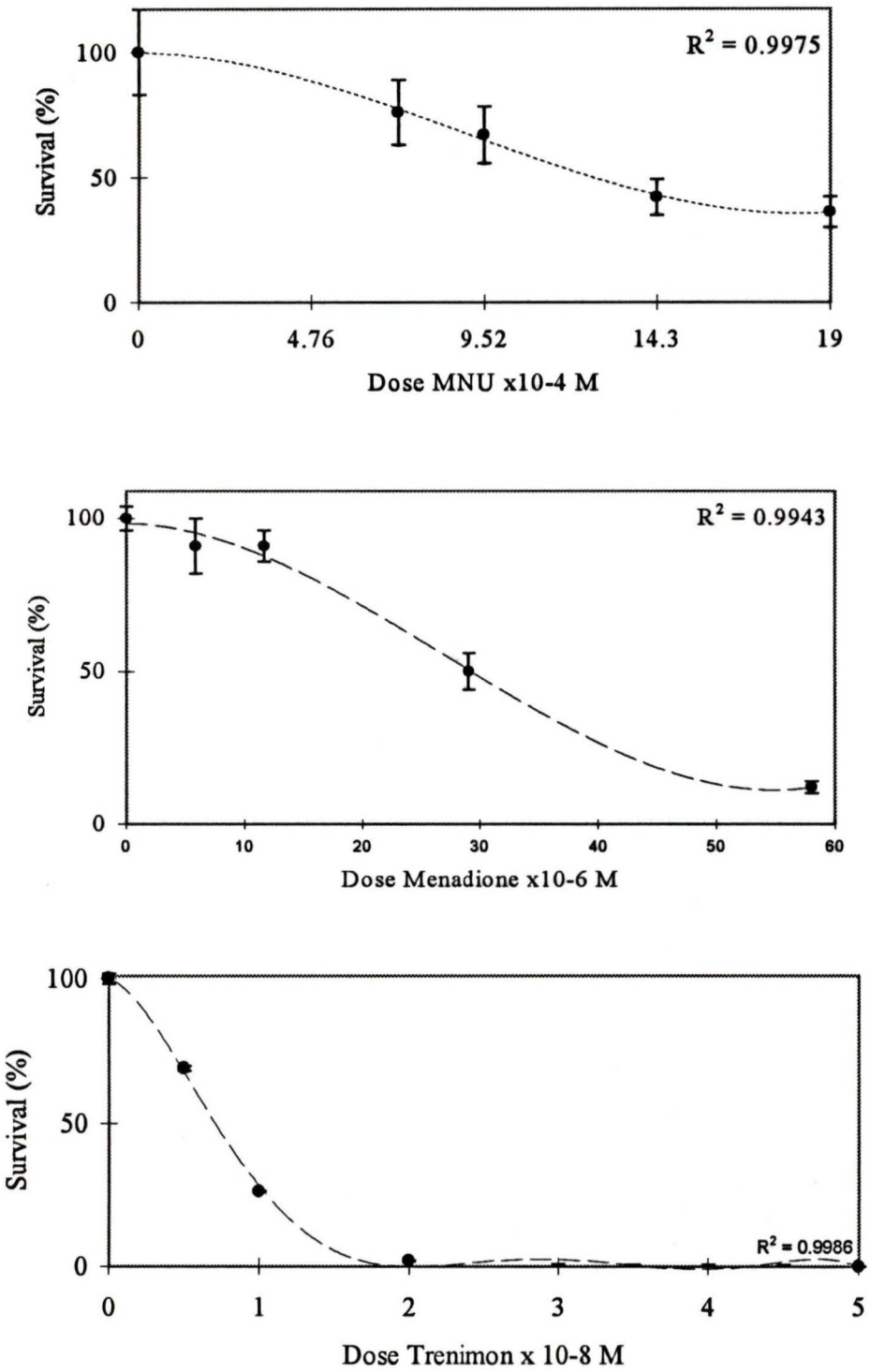


Figure 3.1: R2λLIZ Survival Following 30 minute Chemical Exposure

3.2 Mutant Frequencies Subsequent to MF Exposure

Mutant frequencies (m.f.) from the staggered exposures are reported in Table 3.2. The control group in the blocked experiment with menadione ('blocked' refers to a set of exposures which were conducted at the same time, i.e. menadione and MNU exposures were conducted in experiment 'a', and trenimon and MNU exposures were conducted in experiment 'b') has a m.f. of 5.7×10^{-5}, with a range of $4.7\text{-}7.2 \times 10^{-5}$. The range reflects the error inherent in the method of plaque counting, and is calculated by modifying the number of plaques screened by the standard deviation of the plaque counts. The ethanol control from the blocked experiment with trenimon (experiment 'b') shows a slightly higher m.f. at 7.8×10^{-5}. In both cases, the within field controls have lower mutant frequencies than their no-field counterparts.

Cells exposed to 2.5 and 5 μg/ml of menadione have mutant frequencies of 14×10^{-5}, approximately double the control value. For these menadione concentrations the within field groups have slightly higher mutant frequencies (21×10^{-5} and 18×10^{-5}, respectively).

The high dose of MNU (100 μg/ml) produced a consistently high mutant frequency in the no-field treatments. At 38×10^{-5} and 47×10^{-5} it is apparent that this dose has a six-fold higher m.f. than the control groups. Experiments 'a' and 'b', however, show contradictory results regarding the field exposed MNU groups; the within field m.f. is lower in experiment 'a' and higher in experiment 'b'.

At 0.5 x 10^{-8} M, trenimon gave a m.f. of 16 x 10^{-5}, approximately double that of the ethanol control. At the higher dose the m.f. is 9.9 x 10^{-5}. For both doses of trenimon, the m.f. is lower within the magnetic field than without.

Table 3.2 Mutant Frequencies from Staggered Exposures

Treatment[1]	No Field (0.0002 mT)		Field (3 mT)	
	# Mutants # plaques	**Mutant frequency[2]** & Range	# Mutants # plaques	**Mutant frequency** &Range
Control[a]	22 385187 ± 80413	**5.7** 4.7↔7.2	7 201977 ± 30237	**3.5** 3.0↔4.1
Control (EtOH)[b]	16 206540 ± 13407	**7.8** 7.3↔8.3	14 200130 ± 11893	**7.0** 6.6↔7.4
1 µg/ml Menadione[a]	27 323489 ± 67450	**8.4** 6.9↔11	30 349960 ± 56671	**8.6** 7.4↔10
2.5 µg/ml Menadione[a]	51 377481 ± 87253	**14** 11↔18	18 87136 ± 8825	**21** 19↔23
5 µg/ml Menadione[a]	26 180616 ± 16815	**14** 13↔16	12 67595 ± 15682	**18** 14↔22
20 µg/ml MNU[a]	4 218600 ± 45859	**1.8** 1.5↔2.3	na	na
100 µg/ml MNU[a]	56 145860 ± 22862	**38** 33↔46	32 206780 ± 63181	**15** 12↔22
100 µg/ml MNU[b]	87 184200 ± 2460	**47** 46↔48	646 212620 ± 6053	**304** 295↔313
0.5 x 10^{-8} M Trenimon[b]	32 198760 ± 19458	**16** 15↔18	22 236977 ± 30260	**9.3** 8.2↔11
0.75 x 10^{-8} M Trenimon[b]	22 223280 ± 8683	**9.9** 9.5↔10	7 96240 ± 12193	**7.3** 6.5↔8.3

na, unsuccessful DNA isolation

[1] Mutagen treatments were for 30 minutes. Where applied, the magnetic field was 3 mT for 120 hrs.

[2] Mutant frequency x 10^{-5}

[a] Data from the blocked experiment with menadione.

[b] Data from the blocked experiment with trenimon.

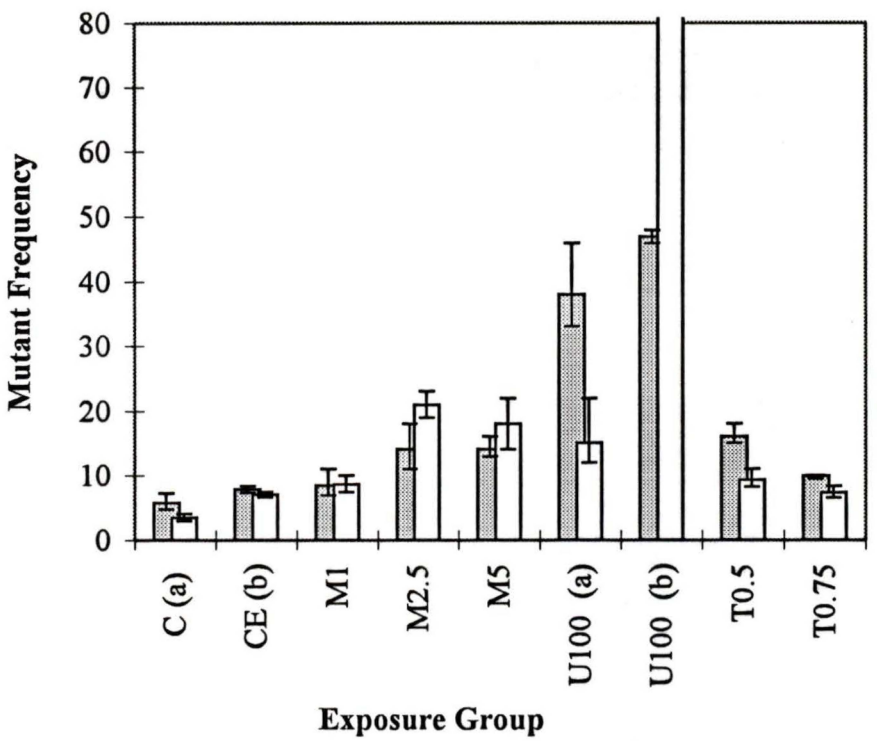


Figure 3.2 Mutant Frequencies from Staggered Exposures. The ordinate of this histogram shows the mutant frequency for each exposure group. Mutant frequencies ($x10^{-5}$) for magnetic field exposed (clear bar) and non-exposed (shaded bar) groups are shown. Abscissa labels are: C, control; CE, 10 µl/ml 100% ethanol; M, menadione (µg/ml); U, MNU (µg/ml); T, trenimon ($x\ 10^{-8}$ M). (a), data from the blocked experiment with menadione.
(b), data from the blocked experiment with trenimon.

Mutant frequencies from the concurrent exposures are shown in Table 3.3. The control m.f.'s are similar to those from the staggered groups.

The menadione 2.5 μg/ml group gave a m.f. of 15 x 10^{-5}, similar to the no-field group from the staggered exposure (m.f. 14 x 10^{-5}). After concurrent application of the field a lower m.f. was found (5.9 x 10^{-5}). The 5 μg/ml dose of menadione gave a no-field m.f. of 6.4 x 10^{-5}, and an in-field m.f. of 5.2 x 10^{-5} (both of which are lower than the corresponding staggered m.f.'s of 14 x 10^{-5} and 18 x 10^{-5} respectively).

At 37 x 10^{-5}, the 100 μg/ml MNU no-field group from experiment 'a' gave a m.f. nearly identical to the corresponding staggered group (38 x 10^{-5}). The in-field groups from the concurrent exposures of both experiments 'a' and 'b', however, have higher m.f.'s than both the no-field group and the corresponding in-field staggered groups. As in the staggered exposure, concurrent application of field and MNU in experiment 'b' gave a much higher m.f. compared to the same exposure group from experiment 'a' (418 x 10^{-5} versus 60 x 10^{-5}).

The no-field trenimon data in Table 3.3 is the same as that in Table 3.2 (for the trenimon experiments a single no-field control group was used for both the staggered-field and the concurrent-field groups). As observed in the staggered data, the m.f.'s with trenimon are lower within the field than without.

Table 3.3 Mutant frequencies from Concurrent Exposures

Treatment[1]	No Field (0.0002 mT)		Field (3 mT)	
	# Mutants # plaques	**Mutant frequency[2]** & Range	# Mutants # plaques	**Mutant frequency** &Range
Control[a]	19 246547 ± 22415	**7.7** 7.1↔8.5	14 176900 ± 26666	**7.9** 6.9↔9.3
Control (EtOH)[a]	6 189415 ± 8202	**3.2** 3.0↔3.3	11 149190 ± 15176	**7.4** 6.7↔8.2
Control (EtOH)[b]	16 206540 ± 13407	**7.8** 7.3↔8.3	14 200130 ± 11893	**7.0** 6.6↔7.4
1 μg/ml Menadione[a]	19 347725 ± 21638	**5.5** 5.1↔5.8	10 137329 ± 11695	**7.3** 6.7↔8.0
2.5 μg/ml Menadione[a]	63 407565 ± 71400	**15** 13↔19	7 119675 ± 12362	**5.9** 5.3↔6.5
5 μg/ml Menadione[a]	26 403802 ± 13053	**6.4** 6.2↔6.7	8 153470 ± 17240	**5.2** 4.7↔5.9
20 μg/ml MNU[a]	14 180583 ± 4350	**7.8** 7.6↔7.9	17 233960 ± 28242	**7.3** 6.5↔8.3
100 μg/ml MNU[a]	134 363870 ± 25951	**37** 34↔40	79 132180 ± 3819	**60** 58↔62
100 μg/ml MNU[b]	87 184200 ± 2460	**47** 46↔48	509 121914 ± 2291	**418** 410↔426
0.5 x 10^{-8}M Trenimon[b]	32 198760 ± 19458	**16** 15↔18	18 323680 ± 39258	**5.6** 5.0↔6.3
0.75 x 10^{-8}M Trenimon[b]	22 223280 ± 8683	**9.9** 9.5↔10	23 233890 ± 18597	**9.8** 9.1↔11

[1] Chemical treatment was for 30 minutes in the presence or absence of a 3 mT magnetic field (total duration 120 hrs)

[2] Mutant frequency x 10^{-5}

[a] Data from the blocked experiment with menadione.

[b] Data from the blocked experiment with trenimon.

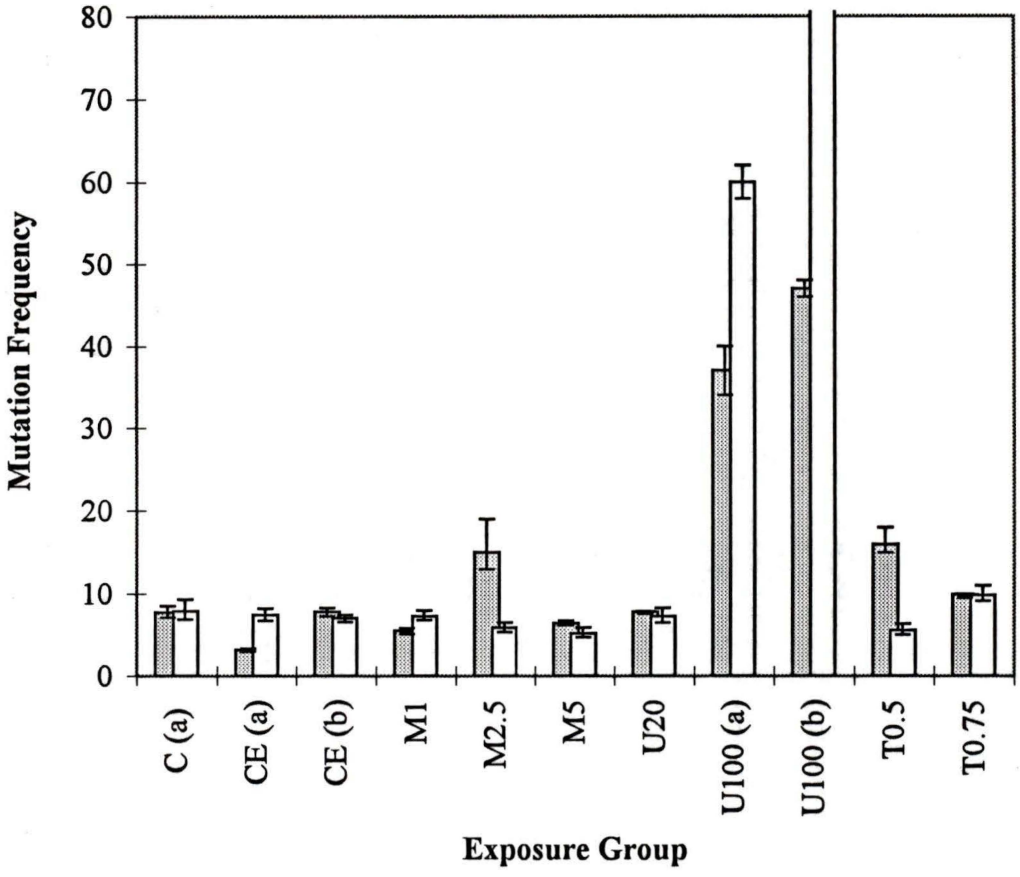


Figure 3.3 Mutant Frequencies from Concurrent Exposure. The ordinate of this histogram shows the mutant frequency for each exposure group. Mutant frequencies ($x10^{-5}$) for magnetic field exposed (clear bar) and non-exposed (shaded) groups are shown. Abscissa labels are: C, control; CE, 10 μl/ml 100% ethanol; M, menadione (μg/ml); U, MNU (μg/ml); T, trenimon ($x\ 10^{-8}$ M). (a), data from the blocked experiment with menadione.
(b), data from the blocked experiment with trenimon.

Comparison of the in-field data from the staggered and concurrent exposures (Table 3.4) reveals that for menadione the concurrent m.f.'s are lower than the staggered. The concurrent MNU groups, however, have higher m.f.'s than the staggered groups. Trenimon does not show a consistent pattern upon comparison of the staggered and concurrent in-field data.

Table 3.4 Staggered Versus Concurrent Mutant Frequencies

Treatment[1]	Staggered	Concurrent
Mutant Frequency[2] & Range from Experiment (a)		
1 ug/ml Menadione	8.6 7.4↔10	7.3 6.7↔8.0
2.5 ug/ml Menadione	21 19↔23	5.9 5.3↔6.5
5 ug/ml Menadione	18 14↔22	5.2 4.7↔5.9
100 ug/ml MNU	15 12↔22	60 58↔62
Mutant Frequency[2] {& Range}from Experiment (b)		
100 ug/ml MNU	304 295↔313	418 410↔426
0.5×10^{-8}M Trenimon	9.3 8.2↔11	5.6 5.0↔6.3
0.75×10^{-8}M Trenimon	7.3 6.5↔8.3	9.8 9.1↔11

[1]Staggered chemical pre-treatments were for 30 minutes. The magnetic field was 3 mT for 120 hrs. Concurrent chemical exposure was for 30 minutes in the presence or absence of a 3 mT magnetic field (total duration 120 hrs)

[2]Mutant frequency x 10^{-5}

(a) Blocked experiment with menadione

(b) Blocked experiment with trenimon

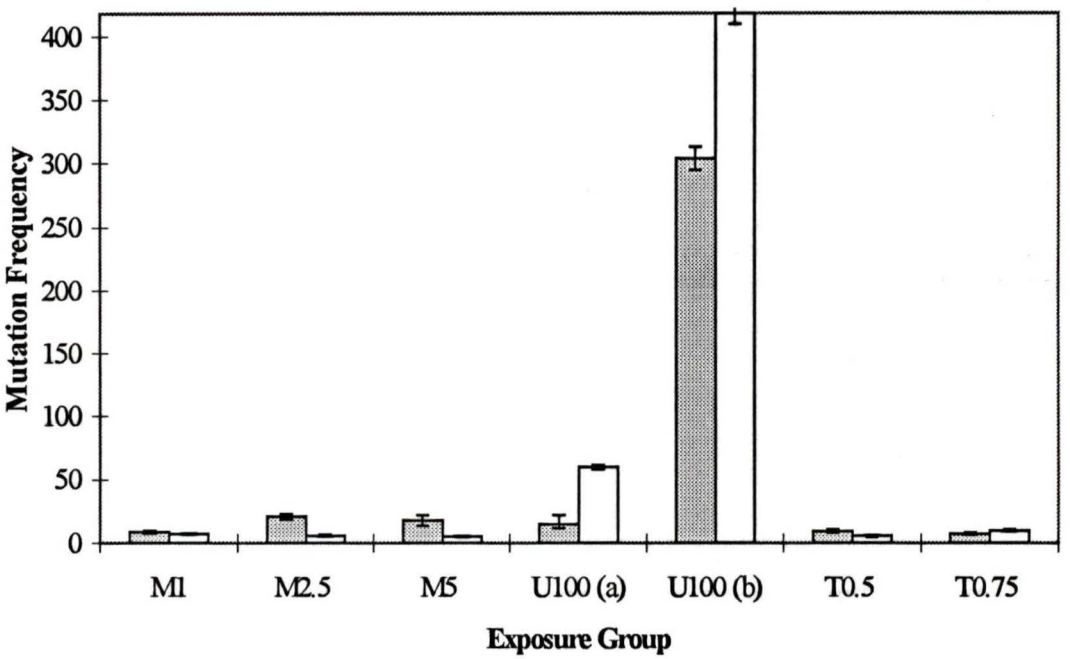


Figure 3.4 Staggered Versus Concurrent Mutant Frequencies. The ordinate of this histogram shows the mutant frequency for each exposure group. Mutant frequencies ($x10^{-5}$) for concurrent (clear bars) and staggered (shaded bars) exposure groups are shown. Abscissa labels are: M, menadione (μg/ml); U, MNU (μg/ml); T, trenimon (x 10^{-8} M). (a), data from the blocked experiment with menadione. (b), data from the blocked experiment with trenimon.

3.3 Statistical Analysis of the Mutant Frequencies: Kastenbaum-Bowman and Traut Tests

The mutant frequencies have been analyzed with two tests of statistical significance. Tables 3.5 - 3.9 show the Kastenbaum-Bowman (KB) analysis, and the last column of each table gives the *P* value as determined by the Traut test (see section 2.9 for details on these tests). For tests of the mutagenicity of a treatment, as in Table 3.5, the *P* value is the probability of finding $\geq x_1$ mutants in the exposure group. For tests of anti-mutagenicity, as in Table 3.6, the *P* value is the probability of finding $\leq x_1$ mutants in the exposure group. An exposure group is considered to have a significantly higher mutation frequency when $x_1 \geq c$ and when $P \leq 0.05$.

The results of the KB and Traut tests for the staggered data are in Table 3.5. While the 2.5 and 5 μg/ml doses of menadione have higher in-field m.f.'s than the no-field groups (see Table 3.2), the increases are insignificant. Specifically, for a significance level of 0.01 at least 4 and 6 more mutants would have had to be recovered in the field exposed 2.5 and 5 μg/ml menadione groups, respectively. The Traut test (final column in Table 3.5) confirms this result.

In the MNU 100 μg/ml groups from experiment 'b', the number of mutants in the field-exposed group (x_1=646) is greater than the rejection number (c=444). This result is not observed in experiment 'a', wherein x_1 (32) < c (63). Comparison of Tables 3.5 and 3.6 reveals that while in experiment 'a' there are significantly fewer mutants in the field-exposed MNU group, in experiment 'b' there are significantly more mutations in the field-exposed MNU group.

Table 3.5 Statistical Analysis of Mutant Frequencies: Testing Mutagenicity/Co-Mutagenicity of MF from Staggered Exposures

Exposure group	Relative proportion of mutants under H_o: $p = N_1 \div N_1+N_2$	Total number of mutants: $n = x_1 + x_2$	Number of mutants in MF exposed group: x_1	Rejection Number, c (α=.05)	Rejection Number, c (α=.01)	Traut Test $P(\geq x_1)$
Control[a]	0.34	29	7	15	17	0.921
Control (EtOH)[b]	0.49	30	14	20	22	0.725
Menadione[a] 1 ug/ml	0.52	57	30	37	39	0.451
2.5 ug/ml	0.19	69	18	20	22	0.063
5 ug/ml	0.27	38	12	16	18	0.248
MNU 100 µg/ml[a]	0.59	88	32	60	63	1.00
100 µg/ml[b]	0.54	733	646	427[1]	444[1]	<0.00001
Trenimon[b] 0.5×10^{-8} M	0.54	54	22	36	39	0.982
0.75×10^{-8} M	0.30	29	7	14	16	0.835

[1]Normal approximation to the binomial as described by McPherson (1990) and Byrkit (1987) was used to generate values of c for n>100.

[a] Data from the blocked experiment with menadione.

[b] Data from the blocked experiment with trenimon.

In the staggered exposures the control groups (with and without ethanol) showed slightly higher mutant frequencies than those within the magnetic field. Table 3.6 shows that these differences are not significant.

The MNU 100 μg/ml treatments from experiment 'a' show a higher number of no-field mutants (x_2=56) than the rejection number (c=48). That the mutant frequency for this treatment is lower within the field than without is confirmed by the Traut test ($P \leq 32$ mutants <0.0001). As previously mentioned, this result is not found in experiment 'b'. At 0.5 x 10^{-8} M trenimon, the number of no-field mutants is equal to the rejection number at a 0.05 level of significance (c=32), but less than $c_{\alpha=0.01}$ (34). The Traut test suggests that the probability of finding less than 22 mutants in the field-exposed group is 0.028. Although the higher trenimon dose also showed a lower m.f. within the field (see Table 3.2), the difference is not significant.

Table 3.6 Statistical Analysis of Mutant Frequencies: Testing Antimutagenicity of MF from Staggered Exposures

Exposure group	Relative proportion of mutants under H_o: $p = N_2 \div N_1+N_2$	Total number of mutants: $n = x_1 + x_2$	Number of mutants in non-MF exposed group: x_2	Rejection Number, c (α=.05)	Rejection Number, c (α=.01)	Traut Test $P(\leq x_1)$
Control[a]	0.66	29	22	24	26	0.143
Control (EtOH)[b]	0.51	30	16	21	23	0.367
Menadione[a] 1 ug/ml	0.48	57	27	35	37	0.621
2.5 ug/ml	0.81	69	51	62	64	0.962
5 ug/ml	0.73	38	26	33	35	0.836
MNU[a] 100 ug/ml	0.41	88	56	44	48	<0.00001
100 µg/ml[b]	0.46	733	87	355[1]	359[1]	1.00
Trenimon[b] 0.5×10^{-8} M	0.46	54	32	32	34	0.028
0.75×10^{-8} M	0.70	29	22	25	27	0.269

[1]Normal approximation to the binomial as described by McPherson (1990) and Byrkit (1987) was used to generate values of c for n>100

[a] Data from the blocked experiment with menadione.

[b] Data from the blocked experiment with trenimon.

The KB and Traut tests of mutagenicity from the concurrent exposures are found in Table 3.7. In experiment 'a', both the control and ethanol-control showed slightly elevated within field m.f.'s (see Table 3.3). Table 3.7 reveals that these elevations are not significant according to the K.B. test. According to the Traut test, however, there are significantly more mutants in the ethanol-field group than in the ethanol group ($P(\geq 11) = 0.01$). Note that in this comparison the no-field m.f. is lower than all other determinations of the spontaneous m.f. found in this study [$x_1=11$ (m.f.$=7.4 \times 10^{-5}$), $x_2=6$ (m.f.$=3.2 \times 10^{-5}$)], for example the no-field m.f. from the ethanol treatment in experiment 'b' is 7.8×10^{-5}, and in-field increases in m.f. are not found in experiment 'b'.

Table 3.7 also shows that the slight in-field elevation in m.f. observed in the menadione 1 μg/ml group is not significant.

In both experiments 'a' and 'b' there are significantly higher in-field m.f.'s for the MNU 100 μg/ml dose. Note that this result is unlike that found in the staggered exposures, where the in-field m.f.'s were only found to be enhanced in experiment 'b'.

Table 3.7 Statistical Analysis of Mutant Frequencies: Testing Mutagenicity/Co-Mutagenicity of MF from Concurrent Exposure

Exposure group	Relative proportion of mutants under H_o: $p = N_1 \div N_1+N_2$	Total number of mutants: $n = x_1 + x_2$	Number of mutants in MF exposed group: x_1	Rejection Number, c (α=.05)	Rejection Number, c (α=.01)	Traut Test $P(\geq x_1)$
Control[a]	0.42	33	14	20	21	0.642
Control (EtOH)[a]	0.44	17	11	12	13	0.010
Control (EtOH)[b]	0.49	30	14	20	22	0.725
Menadione[b] 1 ug/ml	0.28	29	10	13	15	0.224
2.5 ug/ml	0.23	70	7	23	26	0.999
5 ug/ml	0.28	34	8	15	17	0.779
MNU[a] 20 ug/ml	0.56	31	17	23	25	0.625
100 ug/ml[a]	0.27	213	79	61[1]	62[1]	<0.00001
100 ug/ml[b]	0.40	596	509	251	253	<0.00001
Trenimon[b] 0.5×10^{-8} M	0.62	50	18	38	40	0.999
0.75×10^{-8} M	0.51	55	23	35	38	0.528

[1]Normal approximation to the binomial as described by McPherson (1990) and Byrkit (1987) was used to generate values of c for n>100.

[a] Data from the blocked experiment with menadione.

[b] Data from the blocked experiment with trenimon.

Tests of anti-mutagenicity for the concurrent data are reported in Table 3.8. The lower in-field m.f. in the 2.5 μg/ml menadione group is significant according to both statistical test.

The only other significant in-field diminishment of m.f. occurs in the 0.5×10^{-8} M trenimon treatment; at a 0.01 level of significance the number of mutants in the no-field group (x_2=32) is greater than the rejection number (c=28), and according to the Traut test the probability of finding less than 18 mutants in this field-exposed trenimon group is 0.00001. This is a stronger result than that found in the corresponding staggered exposure group (where P=0.028). No such anti-mutagenic effect was found for the higher trenimon dose.

Table 3.8 Statistical Analysis of Mutant Frequencies: Testing Antimutagenicity of MF from Concurrent Exposures

Exposure group	Relative proportion of mutants under H_o: $p = N_2 \div N_1+N_2$	Total number of mutants: $n = x_1 + x_2$	Number of mutants in non-MF exposed group: x_2	Rejection Number, c (α=.05)	Rejection Number, c (α=.01)	Traut Test $P(\leq x_1)$
Control[a]	0.58	33	19	25	27	0.464
Control (EtOH)[a]	0.56	17	6	14	15	0.996
Control (EtOH)[b]	0.51	30	16	21	23	0.368
Menadione[b] 1 ug/ml	0.72	29	19	26	27	0.862
2.5 ug/ml	0.77	70	63	61	63	0.0003
5 ug/ml	0.72	36	26	31	33	0.333
MNU[a] 20 ug/ml	0.44	31	14	19	21	0.469
100 ug/ml[a]	0.73	213	134	>179[1]	>179[1]	0.999
100 ug/ml[b]	0.60	596	87	>401[1]	>401[1]	1.00
Trenimon[b] 0.5×10^{-8} M	0.38	50	32	26	28	<0.00001
0.75×10^{-8} M	0.49	55	22	34	37	0.555

[1]Normal approximation to the binomial as described by McPherson (1990) and Byrkit (1987) was used to generate values of c for n>100.

[a] Data from the blocked experiment with menadione.

[b] Data from the blocked experiment with trenimon.

Table 3.9 summarizes the KB and Traut tests of chemical mutagenicity and the Traut test of anti-mutagenicity. Each exposure group in Table 3.9 is compared to the control (no chemical, no magnetic field) from either the staggered or the concurrent data found in Tables 3.2 and 3.3, respectively. In experiment 'a' the ethanol control appears anti-mutagenic [$P(\leq 6) = 0.014$], though this result is not found in experiment 'b'.

According to the Traut test (but not the KB test), the menadione 1 μg/ml m.f. reported in Table 3.2 is significantly higher than background [$P(\geq 27) = 0.028$]. This result, however, is not reproduced upon analysis of the data in Table 3.3 [$P(\geq 19) = 0.955$].

The doubling of m.f. over background found in the menadione 2.5 μg/ml groups is highly significant in two separate experiments [$P(\geq x_1) < 0.00001$, and $x_1 > c_{\alpha=0.01}$ in each case]. In the 5 μg/ml menadione data a significant increase in m.f. was found in the staggered but not in the concurrent data [$P(\geq 26)<0.00002$ and $P(\geq 26)=0.838$, respectively].

In three independent MNU 100 μg/ml trials the m.f. is significantly higher than background [$P(\geq x_1) < 0.00001$ in each case].

A significant mutagenic response is also apparent in the lower trenimon dose[$P(\geq 32) = 0.0003$]. The higher dose, however, does not show a significant enhancement over the ethanol control.

Table 3.9 Statistical Analysis of Mutant Frequencies: Testing Mutagenicity of the Chemicals

Exposure group	$p = N_1 \div N_1+N_2$	$n = x_1 + x_2$	x_1	c (α=.05)	c (α=.01)	$P(\geq x_1)$, $P(\leq x_1)$
Ethanol[a] (c)	0.43	25	6	16	18	0.994 0.014
Ethanol[b] (c)	0.46	35	16	22	24	0.533 0.566
Menadione[a] 1 μg/ml (s)	0.46	49	27	29	32	0.028 0.983
1 μg/ml (c)	0.59	38	19	28	30	0.955 0.069
2.5 μg/ml (s)	0.49	73	51	44	47	<0.00001 0.999
2.5 μg/ml (c)	0.62	82	63	59	62	<0.00001 0.999
5 μg/ml (s)	0.32	48	26	22	24	<0.00002 0.999
5 μg/ml (c)	0.62	45	26	34	36	0.838 0.212
MNU 20 μg/ml[a] (c)	0.42	33	14	20	21	0.536 0.570
100 μg/ml[a] (s)	0.27	78	56	29	31	<0.00001 1.00
100 μg/ml[a] (c)	0.60	153	134	114[1]	116[1]	<0.00001 1.00
100 μg/ml[b]	0.47	103	87	56[1]	57[1]	<0.00001 1.00
Trenimon[b] 0.5 x 10^{-8} M	0.49	48	32	30	33	0.0003 0.999
0.75 x 10^{-8} M	0.52	38	22	26	28	0.139 0.905

[1, a, b] As in Tables 3.5 - 3.8. (s), (c), (e), vs. within experiment control from Table 3.2 (m.f.=5.7 x 10^{-5}), Table 3.3 (m.f.=7.7 x 10^{-5}), and ethanol control (Table 3.3, m.f.=7.8 x 10^{-5}), respectively.

3.4 Spectra and Statistical Analysis of Sequenced *lac I* Mutations

The mutations identified during this study by sequence analysis of 327 mutant *lacI* transgenes are listed in the Appendix. In this table each treatment group has been assigned a number; for example the control from experiment 'a' is group number "1", and the 0.75 x 10^{-8} M trenimon concurrent-field treatment is group number "15".

The 'occurrence' column in the appended table shows the number of times in the group that a given mutation was recovered. For example, the two base-pair CT deletion at position 947-948 in the *lacI* gene was recovered 10 times in the mutants from the control group in experiment 'a'. Another frequently recovered mutation is the G→A transition at position 56; it is found in 11 of the 15 exposure groups, and occurs 12 times in the trenimon 0.5 x 10^{-8} M treatment (treatment number 10). Entries in Table 3.10 have been corrected for the possibility of clonality, meaning that Appendix occurrences which are > 1 have been reduced to an occurrence of 1. For each treatment group in Table 3.10, then, '*n*' is assumed to be the number of non-clonal sequenced mutations.

In all entries in the Appendix the sequence context shown is that of the coding (non-template) DNA strand.

Most of the mutations identified in this study are single base substitutions (286/327, or 88%). Small deletions (between 2-4 b.p.) are the next most common mutational type (17/327, 5%), followed by larger deletions (11/327 ranging in size between 10-294 b.p., 3 of which have a flanking repeat shown in italics), -1 frameshifts (6/327), +1

frameshifts (3/327), double mutations (2/327), complex mutation (1/327), and insertion (1/327). The insertion of 135 b.p. recovered in treatment number 5 is highly homologous to the known sequence of the rat B2 retroposon (deBoer, *et al.*, 1997).

Table 3.10 Mutational Spectra

Mutation Type	Treatment Number[1] from the Appendix															
	x	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Transition																
G→A	6*	1	1	3	1	13	3	10	6	6	1	1	2	1	1	2
C→T	6	1	0	1	0	9	0	8	5	4	1	2	4	0	0	0
% Total	33.3	25	11.1	28.6	33.3	38.6	18.8	81.8	73.3	100	11.1	30	40	14.3	25	20
% CpG	50	100	100	50	100	4.5	33.3	0	18.2	0	50	66.7	50	100	100	100
A→G	0	0	0	1	0	2	0	0	0	0	2	1	1	1	0	2
T→C	1	1	1	0	0	3	0	0	0	0	3	0	2	0	1	1
% Total	2.8	12.5	11.1	7.1	0	8.8	0	0	0	0	27.8	10	20	14.3	25	30
Transversion																
G→T	4	0	2	1	0	5	4	1	0	0	3	3	1	4	0	1
C→A	0	0	0	1	0	4	0	0	0	0	0	0	2	0	0	3
% Total	11.1	0	22.2	14.3	0	15.8	25	4.5	0	0	16.7	30	20	57.1	0	40
% CpG	75	0	0	50	0	55.6	0	0	0	0	100	33.3	33.3	50	0	100
G→C	0	1	0	1	0	1	1	0	0	0	0	0	0	0	1	0
C→G	5	0	1	0	0	2	1	0	1	0	0	0	1	0	0	0
% Total	13.9	12.5	11.1	7.1	0	5.3	12.5	0	6.7	0	0	0	6.7	0	25	0
% CpG	0	0	0	0	0	33.3	100	0	0	0	0	0	0	0	0	0
A→T	1	0	0	1	2	2	1	0	0	0	1	2	0	0	1	0
T→A	2	0	1	1	0	3	0	2	2	0	1	1	1	0	0	1
% Total	8.3	0	11.1	14.3	66.7	8.8	6.3	9.1	13.3	0	11.1	30	6.7	0	25	10
A→C	4	0	1	0	0	1	1	0	0	0	1	0	0	0	0	0
T→G	1	0	0	1	0	2	0	0	1	0	2	0	0	0	0	0
% Total	13.9	0	11.1	7.1	0	5.3	6.3	0	6.7	0	16.7	0	0	0	0	0
Other																
+1	0	0	1	0	0	1	1	0	0	0	0	0	0	0	0	0
-1	5	1	0	1	0	2	1	0	0	0	0	0	0	0	0	0
del	1	2	1	2	0	6	2	1	0	0	2	0	1	1	0	0
ins	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
complex	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0
double	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
% Total	16.7	50	22.2	21.4	0	17.5	31.3	4.5	0	0	16.7	0	6.7	14.3	0	0
n	36	8	9	14	3	57	16	22	15	10	18	10	15	7	4	10
% clonal	40	60	25	22	25	34	11	27	12	38	40	38	25	61	0	44

[1] Data for treatment 'x' is from the control group in a previous study of the cell line (mutant frequency 5.36 x 10^{-5}; deBoer, personal communication).

The results of the Adams and Skopek Monte-Carlo comparisons of the mutational spectra are presented in Tables 3.11, 3.12 and 3.13. Table 3.11 shows that the spontaneous spectrum recovered from experiment 'a' (group 1) is not significantly different from the spectrum recovered from the cells exposed to the magnetic field alone (group 3; p=0.975). The menadione (groups 5 and 6) and spontaneous (groups x, 1, 2) spectra are not significantly different. Further, the menadione spectrum from the staggered-field exposure (group 6) is not significantly different from the no-field menadione spectrum (group 5; p=0.421).

Table 3.11 Adams & Skopek Analyses of Mutational Spectra: Control and Menadione Treatments

Treatment Number	x	1	2	3	5	6
x	1					
1	0.076	1		p[1]		
2	0.506	0.846	1			
3	0.239	0.975	0.909	1		
5	0.217	0.428	0.714	0.998	1	
6	0.226	0.674	0.973	0.952	0.421	1

[1] All calculations of p (the probability that the two input spectra are the same) are based on 2500 iterations of the Adams and Skopek algorithm.

The no-field MNU spectrum obtained from experiment 'a' (group 7) is significantly different than the spontaneous spectra (groups x, 1, and 2). The experiment 'b' MNU spectrum (group 8) is only different than groups 1 and 2. Further, the MNU spectrum from the staggered-field exposure (group 9) is not significantly different from the no-field MNU spectra (p=0.828 and 0.695).

Table 3.12 Adams & Skopek Analyses of Mutational Spectra: MNU Treatments

Treatment Number	x	1	2	3	7	8	9
7	0.03	0.004	0.004	0.066	1		p[1]
8	0.274	0.019	0.021	0.24	0.858	1	
9	0.149	0.025	0.015	0.194	0.828	0.695	1

Comparison of the trenimon and spontaneous spectra generated within this study reveals no significant differences. Two of the trenimon treatments (group 10, 0.5×10^{-8} M; and group 15, 0.75×10^{-8} M concurrent-field) are significantly different that the spontaneous group 'x' obtained from a previous study.

The no-field 0.5×10^{-8} M trenimon spectrum is not significantly different than either the staggered-field (group 12) or the concurrent-field (group 13) spectra. Neither is the 0.75×10^{-8} M spectrum (group 11) significantly different than the concurrent-field spectrum (group 15, p=0.234).

Table 3.13 Adams & Skopek Analyses of Mutational Spectra: Trenimon Treatments

Treatment Number	x	1	2	3	10	11	12	13	15
10	0.038	0.718	0.91	0.851	1				
11	0.213	0.197	0.66	0.78	0.767	1		p[1]	
12	0.196	0.648	0.637	0.879	0.551	0.521	1		
13	.068	0.164	0.904	0.783	0.829	0.614	0.253	1	
15	0.005	0.203	0.476	0.764	0.413	0.234	0.706	0.186	1

[1] All calculations of p (the probability that the two input spectra are the same) are based on 2500 iterations of the Adams and Skopek algorithm.

Considering the absence of magnetic field induced shifts of mutational spectra, the data from the exposure groups were pooled by chemical exposure. The tests of mutational proportion of the pooled groups are shown in Table 3.14. For each chemical-pool, comparison to the spontaneous data reveals differences in the proportion of mutations belonging to a single mutational class. A group is only included in the pool if it has at least one occurrence of the mutational type under consideration. Thus in Table 3.14 group 1 (the control from experiment 'a') is not included in the spontaneous pool used for comparison to the trenimon data since no G:C→T:A transversions occurred in this group (note that inclusion of group 1 would only augment the difference in proportion reported in Table 3.14).

The trenimon-pool has twice as many G:C→T:A transversions as the spontaneous pool; this type of mutation accounts for 28% of the trenimon mutations and 14% of the spontaneous mutations (P=0.002). The 95% confidence interval (C.I.) for this difference in proportion is 0.05-0.25.

The menadione-pool has 20% more G:C→A:T transitions at non CpG sites than the spontaneous pool (P<<0.0002). The 95% C.I. for this difference in proportion is 0.10-0.30.

The greatest difference in mutational proportion is found in the MNU versus spontaneous comparison. The MNU-pool has considerably more G:C→A:T transitions at non CpG sites than the spontaneous pool (P<<0.0002). 78% of the MNU mutations are of this type, compared to 11% in the spontaneous pool. The 95% C.I. for this difference in proportion is 0.55-0.80.

Table 3.14 Tests of Mutational Proportion

TRENIMON: GC>TA							
	Treatments **10+11+12+ 13+15**	Spontaneous **x + 2+ 3**	π'	$s_{\pi'}$	z^*	***P***	95% C.I
N	124	104	0.2101	0.0517	2.85	0.002	0.05-0.25
n	60	59					
x	17	8					
p	0.283	0.136					
MENADIONE: GC>AT at non-CpG							
	Treatments **5+6**	Spontaneous **x+1+2+3+4**	π'	$s_{\pi'}$	z^*	***P***	95% C.I
N	132	140	0.231	0.0487	4.13	<<0.0002	0.10-0.30
n	73	70					
x	23	8					
p	0.315	0.114					
MNU: GC>AT not at CpG							
	Treatments **7+8+9**	Spontaneous **x+1+2+3+4**	π'	$s_{\pi'}$	z^*	***P***	95% C.I
N	867	140	0.3846	0.0805	8.36	<<0.0002	0.55-0.80
n	47	70					
x	37	8					
p	0.787	0.114					

CHAPTER IV- DISCUSSION

4.1 Mutant Frequencies from Staggered Exposures

By screening over eight million *lacI* transgenes, more than two thousand mutant *lacI* genes (2108) have been identified, from which mutant frequencies have been calculated for cells exposed or not exposed to a 3 mT magnetic field. The question immediately arises as to how comparable the different mutant frequencies are considering the difference in cell survival for the various chemical exposures summarized in Figure 3.1. Of the chemicals studied, trenimon is the most toxic to the R2λLIZ cells on a molar basis (with an LD_{50} of approximately 7.5×10^{-9} M). It is apparent that menadione is more toxic to the R2λLIZ cells than MNU, with approximate LD_{50} values for menadione and MNU at 5 μg/ml (29×10^{-6} M) and 125 μg/ml (11.8×10^{-4} M) respectively. In all cases mutant frequencies have been derived from those cells which have survived the chemical exposure (see section 2.3). Given that the cells were consistently allowed 120 hrs to divide post-chemical exposure, one would expect the number of cell divisions to be similar between exposure groups assuming the growth rate of the cells is constant. This number of cell divisions can be estimated at approximately 2.5 given the change in cell numbers from the time of exposure to the chemicals and the time confluency is reached. Assuming that the rates of cell growth were similar between exposure groups, even where cell numbers varied, then it may be reasoned that the contribution of clonal mutants is relatively constant

between exposure groups, and therefore that the mutant frequencies are comparable. In the absence of growth rate data, however, this assumption must be made explicit.

With one exception, the Kastenbaum-Bowman analyses of the mutant frequency data from the staggered exposures reveal that the hypothesis, H_0, namely that the mutant frequencies in the MF-exposed and non-exposed groups are the same, cannot be rejected since the rejection number of mutants is greater than the number of mutants from MF exposed cells in all cases ($c>x_1$, see Table 3.5). The results of the statistics found in Table 3.5, then, suggest that the mutagenic potentials of the chemicals, menadione, trenimon, and MNU are not significantly enhanced by a MF of 3 mT.

In the R2λLIZ cells the spontaneous (no field, chemical or ethanol) mutant frequency was found to be 5.7×10^{-5} in experiment 'a' and 7.7×10^{-5} in experiment 'b' (see Tables 3.2 and 3.3). According to the Kastenbaum-Bowman test the spontaneous m.f. from experiment 'a' is not significantly higher than that reported by Wyborski *et al.* (4.0×10^{-5}; 1995): $x_1=22$ and $c_{\alpha=0.01}=26$. The difference between the latter and the spontaneous m.f. from experiment 'b' is significant ($x_1=19$, $c_{\alpha=0.01}=19$). These differences suggest that the occurrence of spontaneous mutation may vary by a factor of at least two between subpopulations of R2λLIZ cells.

As described below, treatment of the fibroblasts to the staggered exposure regime did not produce magnetic field enhancements of mutation. According to the radical stabilization hypothesis one would expect magnetic field enhancement of mutation to occur in the menadione and/or trenimon exposure groups. The mutant frequencies of the MF-exposed groups seem slightly higher than the no-field frequencies for

menadione concentrations of 2.5 and 5 μg/ml (see Figure 3.2). From Table 3.5, however, we see that these differences are not significant at α=0.05. The estimated *P*-values suggest that these results may be expected due to chance alone 6% of the time for the 2.5 μg/ml group and 25% of the time for the 5 μg/ml group.

It is clear, however, that exposure of the cells to 2.5 μg/ml menadione caused more than a two-fold induction of mutation over the spontaneous frequency (see Table 3.9). The two tests of the mutagenicity of the 5 μg/ml dose of menadione gave conflicting results; this dose yielded a significantly higher m.f. than the spontaneous in one trial (m.f.=14 x 10^{-5}, P<0.00002), yet failed to increase m.f. in a separate trial (m.f.= 6.4x10^{-5}). The results from the staggered exposures, then, suggest that the 3 mT magnetic field did not enhance the mutagenicity of menadione.

For both doses of trenimon, the mutant frequencies observed from cells exposed to the magnetic field were lower than the m.f. from cells exposed to trenimon alone (see Figure 3.2). The lower mutant frequencies in these field exposed trenimon groups suggest an anti-mutagenic effect of the magnetic field. Cells exposed to 0.5 x 10^{-8} M trenimon had a m.f. of 16 x 10^{-5}, which is significantly higher (Table 3.9; *P*=0.0003) than the within experiment ethanol control m.f. of 7.8 x 10^{-5}, showing that this dose of trenimon is mutagenic. A m.f. of 9.9 x 10^{-5} was observed for the higher dose of trenimon, which is not significantly higher than spontaneous as shown in Table 3.6 (P=0.139), suggesting that this dose is not mutagenic, possibly owing to cell necrosis. The lowering of m.f. in the field exposed trenimon groups is significant for the low dose (see Table 3.6; x_2=32, $c_{\alpha=0.05}$=32, *P*=0.028) and is insignificant for the high dose.

Since the diminishment of m.f. in the magnetic field is only significant in cells exposed to the mutagenic dose of trenimon (0.5×10^{-5} M), it would seem that the magnetic field reduced the mutagenicity of this chemical. The results from the staggered exposures, then, suggest that the low dose of trenimon is mutagenic and that application of the 3mT magnetic field lessened the mutagenicity of this drug.

The results of the two experiments conducted with MNU under the staggered exposure regime gave conflicting results. Table 3.5 describes that in experiment 'b' significantly more mutants occurred in the field-exposed MNU 100 μg/ml group than in the MNU group without field exposure. The results from experiment 'a', however, suggests that the mutant frequency from staggered MNU treatment is significantly smaller inside the MF than outside, since $x_2 > c$ (where $x_2 = 56$ and $c=48$ when $\alpha=0.01$, see Table 3.6). Since the results from the two experiments yielded contradictory results, it is unlikely that the extremely high m.f. derived from the in-field group in experiment 'b' resulted from a magnetic field enhancement of the mutagenicity of MNU. The 100 μg/ml dose of MNU is clearly mutagenic compared to the spontaneous m.f. The three separate determinations of m.f. from cells exposed to this dose of MNU (in the absence of a magnetic field) are in good agreement and range from 33×10^{-5} to 48×10^{-5}, ie. a six-fold induction over the spontaneous m.f. While the results of the staggered MNU exposures show that the 100 μg/ml dose is strongly mutagenic, the influence of the magnetic field on this chemical is uncertain. It is possible that the discrepancy between the results from the two separate staggered exposures with MNU stems from errors in the treatment protocols, either in terms of

dosing errors (low dose given in experiment 'a' and/or high dose given in experiment 'b') or in terms of anomalous clonal expansion in the cells assayed in experiment 'b'.

4.2 Mutant Frequencies from the Concurrent Exposures

Considering the analysis of the menadione and trenimon mutant frequencies from the concurrent exposures, it is evident that according to the Kastenbaum-Bowman tests, the null hypothesis that the mutant frequencies in the MF-exposed and non-exposed groups are the same, cannot be rejected since the rejection number of mutants is greater than the number of mutants from MF exposed cells in all cases ($c>x_1$, see Table 3.7). These results, then, suggest that the mutagenic potentials of menadione and trenimon are not enhanced by a MF of 3 mT.

Contrary to the lack of field influence in the menadione 2.5 μg/ml in-field group from the staggered exposures, there is a significant reduction in the m.f. of this dose within the field from the concurrent exposures (Table 3.8; $x_2=63$, $c_{\alpha=0.01}=63$, $P=0.0003$). The m.f.'s from the menadione concurrent-field groups are lower than the staggered-field groups (see Figure 3.4). As mentioned in the introduction (section 1.7) one would expect direct influences of the magnetic field on chemical mutagenicity to be revealed in the staggered exposures wherein the chemical is allowed time to enter the cells in the absence of the field. During the staggered exposure there was no possibility of a confounding effect of the MF on the plasma membrane (since the chemical was washed from the culture media and the media was replaced before the MF exposure began). In the concurrent exposures, however, there is the confounding possibility

that the magnetic field could influence the entry of the chemical into the cells. Taken together, then, these results suggest that the magnetic field cannot directly influence the mutagenicity of the menadione via a mechanism such as radical stabilization, but may restrict the entry of menadione into the cell. Table 3.9 shows that the 2.5 μg/ml dose of menadione is mutagenic ($P<0.00001$), causing nearly a two-fold higher m.f. than the control m.f. of 7.7×10^{-5}. The combined results from the staggered and concurrent exposures, then, suggest that the 3 mT magnetic field did not enhance the mutagenicity of menadione.

For both doses of trenimon, the mutant frequencies observed from cells concurrently exposed to the magnetic field were lower than the m.f. from cells exposed to trenimon alone (see Figure 3.3). As with the staggered exposures, the lower mutant frequencies in these field-exposed trenimon groups suggest an anti-mutagenic effect of the magnetic field. The lowering of m.f. in the field exposed trenimon groups is significant for the low dose (see Table 3.8; $x_2=32$, $c_{\alpha=0.01}=28$, $P<0.00001$) and is insignificant for the high dose. Since the field-exposed cells had significantly lower m.f.'s than their non-exposed partners after both staggered and concurrent exposure, the reduction in m.f. cannot be explained solely by a MF-induced alteration in the permeability of the cell membrane to ethanolic trenimon. The explanation of this result with trenimon - that the MF reduced the activity of the chemical - must also take into consideration the finding that the field did not give the same result for menadione. Given that both chemicals can experience a two electron reduction by DT-diaphorase, and that this metabolic pathway is thought to be more important for trenimon than for menadione induced mutagenesis (see chapter 1.6), it is therefore possible that the MF

inhibited the activity of this enzyme. The combined results from the staggered an concurrent exposures are in agreement, suggesting that the low dose of trenimon is mutagenic and that application of the 3mT magnetic field lessened the mutagenicity of this drug.

In both experiments 'a' and 'b' there are significantly higher in-field m.f.'s for the MNU 100 μg/ml dose. Note that this result is unlike that found in the staggered exposures, where the in-field m.f. (304×10^{-5}) was only enhanced in experiment 'b'. It is also apparent that the magnitude of the m.f. increase within the field is much higher in experiment 'b' (m.f.=418×10^{-5}) than in experiment 'a' (m.f.=60×10^{-5}). Since the in-field m.f.'s for both the staggered and concurrent MNU exposure groups are much higher in experiment 'b' than in experiment 'a', and since the in-field increase was only observed in one of the two staggered exposures conducted, it is unlikely that the magnetic field directly influenced the mutagenicity of this chemical. There are two other explanations which fit the data. First, if the magnetic field increased the permeability of the cell membranes to MNU, then (as observed) one would expect field enhancements in the concurrent groups. Second, it is possible that the elevated m.f.'s in these groups (especially in experiment 'b' wherein the m.f.'s are highest) were caused by an abnormally high contribution of clonally derived mutants. While the results of the concurrent MNU exposures in the absence of the magnetic field show that the 100 μg/ml dose is strongly mutagenic (see Table 3.9), the effect of the magnetic field on MNU mutagenicity is unclear.

The influence of the 3 mT magnetic field on mutant frequency may be summarized by the following observations. The magnetic field did not increase the frequency of mutation in the control cells, that is, in cells grown with or without ethanol. The mutagenic potentials of menadione and trenimon were not enhanced by a MF of 3 mT. However, it is possible that the magnetic field restricted the entry of menadione into the cell, as evidenced by the suppression of m.f. upon concurrent application of the field. The results from the staggered and concurrent exposures suggest that the low dose of trenimon is mutagenic and that application of the 3mT magnetic field lessened the mutagenicity of this drug. While the influence of the MF on MNU mutagenesis is uncertain, it is clear that the field did not consistently enhance mutation in the staggered exposures, and that the in-field groups from experiment 'b' were abnormally high, suggesting that the results with this chemical are best explained by membrane and/or clonal influences.

There are several other possible explanations for the observed differences in mutant frequencies between exposure groups and between exposure protocols. The cytotoxicity, and presumably genotoxicity, of menadione and trenimon are dependent on NAD(P)H-cytochrome P450 reductase. Cell populations with reduced reductase activity have been shown to be resistant to menadione (Sawada, et al., 1991). It is possible that the diminished m.f.'s in the concurrently field-exposed menadione groups and in the field-exposed trenimon groups, are due to different levels of activity of this enzyme between subpopulations of R2λLIZ cells. Similarly, variation among fibroblasts in the liberation of reactive oxygen in the cell and/or in the ability of the reactive species to attack DNA could account for the observed differences in mutant

frequency. Variation could also stem from subpopulation differences in antioxidant defense (in terms of free radical scavenging enzymes) (Collins, 1994), or in DNA repair capabilities.

It is also possible that one or both of the radicals resultant from the reduction of menadione or trenimon can undergo rapid spin relaxation, rendering them insensitive to the magnetic field (Scaiano, 1995). This consideration underscores the need for more chemicals to be tested in this system. Further, it could be that the field strength tested (3mT) is under the $B_{1/2}$ value (the field strength at which 50% of the maximum radical-stabilization effect can be achieved) for menadione and trenimon, pointing to the need for testing stronger magnetic fields in this system.

It was noted that the mutant frequencies for the middle and high dose of menadione are similar. *In vitro* experiments measuring DNA damage by Fenton reactions ($Fe^{2+} + H_2O_2 \rightarrow \bullet OH + H_2O$) suggest that the maximum extent of DNA nicking occurs at low concentrations of hydrogen peroxide, with high concentrations suppressing about one half of the damage (Imlay & Linn, 1988). This may also be the case for menadione induced production of hydrogen peroxide.

To be sure that an applied magnetic field is not co-mutagenic, a broad range of compounds and MF strengths need to be tested. It is certain, however, that these experiments suggest that a prolonged 3 mT 60 Hz MF exposure, whether alone or in conjunction with menadione, trenimon or MNU, is unable to significantly produce or enhance a mutagenic effect under the test conditions employed. To rule out the possibility of a MF as an initiator of mutation, however, a wider range of field

strengths, exposure times, frequencies and chemical treatment modalities need to be explored.

It may be that a co-mutagenic effect of a MF, if it exists for any compound, may only be revealed upon sequence analysis of recovered mutants. Recent studies showing that MF induces a stress response in some cells (Goodman *et al.*, 1994b; 1994c) may entail that DNA repair pathways are influenced by a field, which could for example account for the suppression of mutant frequency for trenimon reported in this study, or for the levelling of mutant frequencies between MF exposed and non-exposed groups. It is unlikely that the 3 mT MF-induced 'suppression' of mutant frequency observed for the staggered MNU and trenimon treatments and the concurrent trenimon treatment can be explained by such a response, however, since the gene expression changes detected in the abovementioned studies occur only at very low field strengths (on the order of 10-100 μT, with no response at higher field strengths). It is also unlikely that the minute background field level of 0.2 μT in the control incubator influenced gene expression in the control cells, since this flux density is much lower than the window wherein such changes reportedly occur. Assuming that the observed frequencies do not involve gene expression changes, then analysis of the sequenced mutations should help to define the interplay, if any, between the 3 mT MF and the test chemicals. Sequence information could reveal as yet hidden co-mutagenic effects in the form of shifts in mutational spectra between MF exposed and non-exposed groups, and provide information about the mechanism of mutation in the different exposure groups.

4.3 Mutational Spectra at the *lac I* Locus

In total 327 mutations were characterized by automated laser fluorescence (ALF, Pharmacia) DNA sequencing. A spontaneous spectrum was obtained from a previous study of the cell line (group 'x', deBoer & Glickman, unpublished). The Adams and Skopek analyses did not reveal spectral differences between the field-exposed and non-exposed groups (see Tables 3.11 through 3.13).

Compared to the spontaneous spectra determined from these experiments (groups 1 and 2), only the MNU spectra (groups 7, 8 and 9) were found to be significantly different ($p \leq 0.025$ in all comparisons, see Table 3.12). Examination of the mutational spectra corrected for the possibility of clonality (Table 3.10) reveals that G:C → A:T transition is the predominant mutation in the MNU spectra (39/53 independent mutations are of this type, only two of which occur at CpG sites). The induction of G:C → A:T transitions is expected of MNU and is thought to result from the mispairing of O^6-methylguanine with thymidine during DNA synthesis (Richardson *et al.*, 1987). It could further be argued that the attack of MNU on a base will depend on the degree of methylation of that base or of the surrounding bases, and therefore that MNU would avoid CpG sites which have 5-methylcytosine residues. Indeed, the test of mutational proportion (Table 3.14) reveals that the pooled-MNU groups have at least 55% more of G:C → A:T transitions at non-CpG sites than the spontaneous groups (95% C.I.=0.55-0.88).

While the Adams and Skopek analyses of the menadione and trenimon spectra did not reveal significant differences between either the in-field or the spontaneous groups (see Tables 3.11 and 3.13), the tests of mutational proportion show that the chemical-pools

have a greater proportion of a single base substitution (see Table 3.14). The menadione groups have more G:C → A:T transitions at non-CpG sites than the spontaneous groups (95% C.I.=0.1-0.3). Given that this type of transition is a common mutation recovered upon Fe^{2+} mutagenesis (and thus upon exposure to oxygen radicals; McBride, 1991), it is possible that the transitions found in the menadione pool resulted from oxidative stress. Although the promutagenic lesion for oxygen mediated G:C → A:T transition is unknown, Wagner *et al.* (1992) have characterized several types of oxidative damage targeted to deoxycytidine, and argue that 5,6-dihydroxy-5,6-dihydro-2'-deoxyuridine can mispair with deoxyadenine, leading to G:C→A:T transition.

Since the occurrence of G → T transversions is similar in the spontaneous and menadione data it must be concluded that there is no evidence from this data set for 8-oxoG damage resultant from menadione exposure. This is the expected lesion resultant form the attack of the hydroxyl radical on guanine (Urios *et al.*, 1995). This result is in agreement with previous study of menadione where the induction of Trp^+ revertants in a mutY DNA-glycosylase deficient strain of E.coli was not increased by menadione (Urios *et al.*, 1995).

The expected increases over background of G→ C and G → T base substitutions after menadione exposure were not observed. It is possible that the lesions which produce these mutations were in fact formed (eg. 8-hydroxyguanosine lesions for the G → C transversion), but if so then they did not overwhelm the repair capabilities of the R2λLIZ cells.

Comparison of the pooled trenimon data set to the spontaneous revealed that the former has twice as many G:C →T:A transversions (p=0.002, 95% C.I.=0.05-0.25), suggesting that this type of mutation can be induced by trenimon. As described in the Introduction (section 1.6), trenimon can be reduced by NADP(H) cytochrome P-450 reductase to yield a semiquinone radical, which in turn can be oxidized to reform trenimon and form the superoxide anion radical $O_2^{\bullet-}$ (Silva, *et al.*, 1992). Through Fenton and Haber-Weiss chemistry, the latter species is involved in hydroxyl radical production (Nutter, *et al.*, 1992; McBride, *et al.*, 1991), and thus in the formation of 8-oxoGuanine (8-oxoG) lesions (Tchou and Grollman, 1993). Since 8-oxoG preferentially mispairs with adenine, resulting in G:C →T:A transversion, it is possible that the disproportion of these mutations in the trenimon pool was caused by the abovementioned reactions.

In closing, the analyses of the mutational spectra has not revealed any differences between the field-exposed and non-exposed groups. Only the MNU spectra were found to be significantly different than the spontaneous spectra according to the Adams and Skopek test, owing to a large number of G:C → A:T transitions in the MNU data sets. The finding of base substitution disproportions in the pooled chemical groups (G:C →A:T at non-CpG sites for MNU and menadione, and G:C →T:A for trenimon) provides some insight into the mechanisms of mutagenesis of each chemical. As expected, the occurrence of G:C →A:T transitions in the MNU pool supports the known alkylative mechanism of mutagenesis of this chemical. The occurrence of this same type of mutation in the menadione pool, while less open to mechanistic explanation, is not incompatible with the paradigm of menadione induced oxidative

stress. Last, the weak disproportion of G:C →T:A transversions in the trenimon pool points to the oxidative metabolic pathway of this drug as a contributor to trenimon induced mutagenesis, probably by the eventual production of hydroxyl radicals.

CHAPTER V - CONCLUSIONS

This project aimed to investigate the potential influence of a magnetic field on the process of mutagenesis and co-mutagenesis. No enhancements of mutagenicity were found for the test chemicals, menadione and trenimon, in the presence of the magnetic field. Consistent enhancements of MNU mutagenicity were not found since only one of two staggered experiments with MNU showed a higher in-field mutant frequency. Neither is there evidence for a mutagenic or anti-mutagenic influence of the MF in the absence of chemical exposure.

Contrary to expectation, evidence for a magnetic field induced suppression of the mutagenicity of trenimon was found. However, since this observation is based on only one experiment, it is premature to say whether this is a real interaction between the magnetic field and trenimon.

Some indirect evidence was found to suggest an influence of the magnetic field on membrane permeability. Specifically, there is reason to believe that the magnetic field inhibited passage of menadione into the cells and augmented that of MNU. However, without specifically testing this phenomenon and quantifying the traversal of each drug into the fibroblasts, it must be recognized that the influence of the magnetic field on the cell membrane is uncertain.

It was confirmed that the chemicals themselves are mutagenic. MNU gave the highest induction over background (approximately six-fold). Menadione and trenimon yielded weaker mutagenic responses, with exposed groups having about a two-fold higher mutant frequency than controls.

Sequence analysis of sub-sets of the recovered mutations has not revealed pronounced spectral differences between the magnetic field exposed and non-exposed groups. Pooling of the data allowed tests of mutational proportion to be conducted, which have revealed disproportions between the chemical and the spontaneous spectra in the occurrence of G:C → A:T transitions (for MNU and menadione) and G:C → T:A transversions (for trenimon).

The following suggestions are intended as a guide for future research on the influence of magnetic fields on mutation, in this or other transgenic systems, under the radical stabilization hypothesis. First, without knowing the $B_{1/2}$ value for the radicals produced by a given mutagen, it is clear that the strength of the magnetic field tested should be varied. Given that magnetic field mutagenic enhancements of trenimon and menadione were not detected in this study, future studies on these chemicals should employ a stronger magnetic field.

The disparity between the staggered and the concurrent results point to the possibility of a magnetic field influence on membrane permeability. Future studies on the mutagenicity and co-mutagenicity of magnetic fields, then, would benefit from the testing of magnetic field induced changes to the cell surface, for example by the 'cell surface chromatography' approach of Goodman *et al.* (1986). Similarly, to rule out the confounding possibility of magnetic field alterations to the production of specific transcripts (especially to DNA repair enzymes) future studies with the R2λLIZ cells (or other transgenic systems) and magnetic fields should aim to detect such alterations.

Future research would also benefit from the testing of different chemicals (for example nickel compounds, or other free-radical producers) or higher concentrations of the test chemicals used in this study. At the concentrations tested it was observed that trenimon is very toxic to the rat fibroblasts, producing only a weak mutagenic response. It would therefore be interesting to conduct an experiment with a cell line which can withstand a greater dose of trenimon. One candidate worth testing, for example, is the transgenic mouse fibroblast cell line (Stratagene, LaJolla, CA).

Last, since the method of detecting mutations employed in this system precludes the detection of large deletions and chromosomal damage, the use of transgenic cell lines for the study of free-radical induced (and MF enhancement of) DNA alterations would benefit from the inclusion of other assays for DNA damage, specifically for strand breakage, such as the 'comet' or sister chromatid exchange tests, in conjunction with the *lacI* transgene assay.

In conclusion, these experiments aimed at detecting mutagenic enhancements of free-radical producing chemicals have not found convincing evidence for the phenomenon of radical stabilization in the rat embryo fibroblast transgenic system. It must be noted, however, that these experiments do not pretend to be exhaustive in their ability to detect such a phenomenon. The relevance of radical stabilization to the process of mutagenesis will only be revealed upon further experimentation.

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APPENDIX: *lac I* mutations

Treatment[1]	Occurrence	Position[2]	Mutation[3]	Template Sequence Context[4]	A.A.[5] Change
1	1	282, 284	- C, C>T	AAA TCT CGC GCC	na
Control (a)	1	54	T>C	GAT GTC GCA	V>A
	4	56	G>A	GTC GCA GAG	A>T
	1	201	G>C	GCG GGC AAA	G>A
	1	202-205	del CAAA	GGC AAA CAG	na
	1	329	C>T	GAA CGA AGC	R>stop
	1	559	- G	GCA TTG?GGT	na
	10	947-948	del CT	CAA CTC TCT	na
2	4	56	G>A	GTC GCA GAG	A>T
Control	1	119	T>C	GTT TCT GCG	S>P
(Ethanol)	1	141	T>A	AAA GTG GAA	V>E
(b)	1	176	A>C	CCC AAC CGC	N>H
	1	183-199	del 17 b.p.	CGC GTG ...GCG GGC	na
	1	186	C>G	GTG GCA CAA	A>G
	1	466	+ A	TTA...TTT	na
	1	790	G>T	GCA ATG CGC	M>I
	1	1005	G>T	AAA AGA AAA	R>I
3	1	20	G>C	AGG GTG GTG	na
3 mT (a)	2	30	T>A	AAT GTG AAA	V>E
	1	81	A>G	TAT CAG ACC	Q>R
	1	82	G>T	TAT CAG ACC	Q>H
	1	93	G>A	TCC CGC GTG	R>H
	1	107-129	del 22 b.p.	GCC AGC...ACG CGG	na
	1	176	- A	CCC A?AC CGC	na
	1	202-205	del CAAA	GGC AAA CAG	na
	1	210	C>A	CAG TCG TTG	S>stop
	3	270	C>T	GTC GCG GCG	A>V
	1	369	T>G	AAT CTT CTC	L>R
	2	631	G>A	GGC TGG CAT	W>stop
	1	702	G>A	TCC GGT TTT	G>D
	1	1004	A>T	AAA AGA AAA	R>stop
4	2	56	G>A	GTC GCA GAG	A>T
3 mT (b)	1	968	A>T	GTG AAG GGC	K>stop
	1	978	A>T	AAT CAG CTG	Q>L

[1,2,3, 4, 5] (see end of table for explanation).

lac I mutations (continued)

Treatment	Occurrence	Position	Mutation	Template Sequence Context	A.A. Change
5	3	30	T>A	AAT GTG AAA	V>E
Menadione	1	49	C>A	TTA TAC GAT	Y>stop
2.5 µg/ml	3	56	G>A	GTC GCA GAG	A>T
(a)	2	72	T>C	GGT GTC TCT	V>A
	1	75	C>T	GTC TCT TAT	S>F
	1	77	ins 135 b.p	GTC TCT T...AT CAG ACC	na
	2	78	A>C	TCT TAT CAG	Y>S
	1	83	A>G	CAG ACC GTT	T>A
	1	89	T>C	GTT TCC CGC	S>P
	2	90	C>T	GTT TCC CGC	S>F
	1	93	G>T	TCC CGC GTG	R>L
	1	101	A>G	GTG AAC CAG	N>D
	1	109-132	del 24 b.p.	GCC AGC...CGG GAA	na
	1	143	G>T	GTG GAA GCG	E>stop
	1	167	T>C	AAT TAC ATT	Y>H
	1	167	T>G	AAT TAC ATT	Y>D
	1	177	A>T	CCC AAC CGC	N>I
	3	185	G>A	GTG GCA CAA	A>T
	1	187-189	del ACA	GTG GCA CAA CAA	na
	1	191	C>G	CAA CAA CTG	Q>E
	2	197	G>A	CTG GCG GGC	A>T
	1	201	G>A	GCG GGC AAA	G>D
	1	222	G>A	ATT GGC GTT	G>D
	1	260	C>T	TCG CAA ATT	stop
	1	273	C>A	GCG GCG ATT	A>E
	1	282	C>T	AAA TCT CGC	Q>stop
	1	284	C>G	TCT CGC GCC	R>G
	1	316-337	del 22 b.p.	*G TCG A...GGC GTC GA*	na
	1	318	C>A	GTG TCG ATG	S>stop
	1	364	- C	GTG CAC AAT	na
	1	373-424	del 52 b.p.	CTC GCG...GAT GCC	na
	1	374-667	del 294 b.p.	*GCG CAA CG*...ATA *GCG GAA CG*	na
	1	377	C>T	GCG CAA CGC	Q>stop
	1	506	+ T	ATT.?.TTC	na
	3	518	G>A	CAT GAA GAC	E>stop
	4	525	G>A	GAC GGT ACG	G>D
	3	537	G>A	CTG GGC GTG	G>D

lac I mutations (continued)

Treatment	Occurrence	Position	Mutation	Template Sequence Context	A.A. Change
5	1	569	C>T	CAG CAA ATC	Q>stop
Menadione	3	582	T>A	CTG TTA GCG	L>stop
2.5 µg/ml	1	586	- G	GCG?GGC	na
(a)	2	631	G>A	GGC TGG CAT	W>stop
	1	659	C>T	ATT CAG CCG	Q>stop
	3	681	G>A	GAA GGC GAC	G>D
	1	782	G>A	CTG GGC GCA	G>S
	1	783	G>A	CTG GGC GCA	G>D
	1	783	G>T	CTG GGC GCA	G>V
	1	788	A>T	GCA ATG CGC	M>L
	2	790	G>T	GCA ATG CGC	M>I
	1	810	G>C	TCC GGG CTG	G>A
	1	831	T>A	GAT ATC TCG	I>N
	3	842	G>A	GTG GGA TAC	G>R
	3	843	G>A	GTG GGA TAC	G>E
	2	888	C>T	TTA ACC ACC	T>I
	1	928	C>A	ACC AGC GTG	S>R
	1	947-948	del CT	CAA CTC TCT	na
	1	999	T>G	CTG GTG AAA	V>G
	2	1011	C>T	AAA ACC ACC	T>I
6	1	41	A>T	GTA ACG TTA	T>S
Menadione	2	43	- G	GTA ACG TTA	na
2.5 µg/ml	1	158	G>T	GCG GAG CTG	E>stop
+ 3mT	1	164, 166	A>G, T>G	CTG AAT TAC	N>G
Staggered	1	187-189	del ACA	GTG GCA CAA CAA	na
(a)	1	200	G>A	GCG GGC AAA	G>S
	1	204	A>C	GGC AAA CAG	K>T
	1	284	C>G	TCT CGC GCC	R>G
	1	306	G>T	GCC AGC GTG	S>I
	1	575	G>C	ATC GCG CTG	A>P
	1	587	G>T	GCG GGC CCA	G>C
	1	611	+ A	GCG C...GT CTG	na
	1	681	G>A	GAA GGC GAC	G>D
	1	794	G>A	CGC GCC ATT	A>T
	1	842	G>T	GTG GGA TAC	G>stop
	2	947-948	del CT	CAA CTC TCT	na
7	1	54	T>A	GAT GTC GCA	V>D
MNU	1	109-132	del 24 b.p.	GCC AGC...CGG GAA	na
100 µg/ml	1	140	G>A	AAA GTG GAA	V>M
(a)	1	162	T>A	GAG CTG AAT	L>Q

lac I mutations (continued)

Treatment	Occurrence	Position	Mutation	Template Sequence Context	A.A. Change
7	1	173	C>T	ATT CCC AAC	P>S
MNU	2	174	C>T	ATT CCC AAC	P>L
100 μg/ml	2	197	G>A	CTG GCG GGC	A>T
(a)	1	201	G>A	GCG GGC AAA	G>D
	1	222	G>A	ATT GGC GTT	G>D
	1	258	C>T	CCG TCG CAA	S>F
	2	569	C>T	CAG CAA ATC	Q>stop
	1	588	G>T	GCG GGC CCA	G>V
	1	631	G>A	GGC TGG CAT	W>stop
	1	659	C>T	ATT CAG CCG	Q>stop
	2	681	G>A	GAA GGC GAC	G>D
	1	702	G>A	TCC GGT TTT	G>D
	1	710	C>T	CAA CAA ACC	Q>stop
	2	782	G>A	CTG GGC GCA	G>S
	3	783	G>A	CTG GGC GCA	G>D
	2	842	G>A	GTG GGA TAC	G>R
	1	888	C>T	TTA ACC ACC	T>I
	1	959	C>T	GGC CAG GCG	Q>stop
8	1	42	C>T	GTA ACG TTA	T>M
MNU	3	56	G>A	GTC GCA GAG	A>T
100 μg/ml	1	75	C>T	GTC TCT TAT	S>F
(b)	1	84	C>T	CAG ACC GTT	T>I
	1	87	T>A	ACC GTT TCC	V>D
	1	90	C>T	GTT TCC CGC	S>F
	1	140	G>A	AAA GTG GAA	V>M
	1	188	C>G	GCA CAA CAA	Q>E
	1	201	G>A	GCG GGC AAA	G>D
	1	525	G>A	GAC GGT ACG	G>D
	1	558	T>G	GCA TTG GGT	L>W
	1	681	G>A	GAA GGC GAC	G>D
	1	702	G>A	TCC GGT TTT	G>D
	1	719	C>T	ATG CAA ATG	Q>stop
	1	990	T>A	CCC GTC TCA	V>D
9	3	90	C>T	GTT TCC CGC	S>F
MNU	2	186	C>T	GTG GCA CAA	A>V
100 μg/ml	1	206	C>T	AAA CAG TCG	Q>stop
+ 3mT	1	681	G>A	GAA GGC GAC	G>D
Staggered	1	702	G>A	TCC GGT TTT	G>D
(b)	1	783	G>A	CTG GGC GCA	G>D
	1	842	G>A	GTG GGA TAC	G>R

lac I mutations (continued)

Treatment	Occurrence	Position	Mutation	Template Sequence Context	A.A. Change
9 (continued)	3	843	G>A	GTG GGA TAC	G>E
	2	918	G>A	CTG GGG CAA	G>E
	1	953	C>T	TCT CAG GGC	Q>stop
10 Trenimon 0.5 x 10^{-8}M (b)	1	556-557	AT>C	GCA TTG	na
	1	8-	T>C	TGATAGCGC	na
	1	39	T>A	CCA GTA ACG	V>E
	1	41	A>G	GTA ACG TTA	T>A
	12	56	G>A	GTC GCA GAG	A>T
	1	83	A>G	CAG ACC GTT	T>A
	1	86	G>T	ACC GTT TCC	V>F
	1	87	T>C	ACC GTT TCC	V>A
	1	93	G>T	TCC CGC GTG	R>L
	1	176	A>C	CCC AAC CGC	N>H
	1	245-295	del 51 b.p.	GCC *CTG* ...CAA *CTG*	na
	1	294-303	del 10 b.p.	GAT CAA ...GCC AGC	na
	1	374	G>T	CTC GCG CAA	A>S
	2	411	T>G	CCG CTG GAT	L>R
	1	465	T>G	GCG TTA TTT	L>stop
	1	468	T>C	TTA TTT CTT	F>S
	1	569	C>T	CAG CAA ATC	Q>stop
	1	635	A>T	CAT AAA TAT	K>stop
11 Trenimon 0.75 x 10^{-8}M (b)	7	56	G>A	GTC GCA GAG	A>T
	1	183	T>A	CGC GTG GCA	V>E
	1	221	G>T	ATT GGC GTT	G>C
	1	278	A>T	ATT AAA TCT	K>stop
	1	329	C>T	GAA CGA AGC	R>stop
	1	350	A>T	TGT AAA GCG	K>stop
	1	374	G>T	CTC GCG CAA	A>S
	1	485	C>T	GAC CAG ACA	Q>stop
	1	790	G>T	GCA ATG CGC	M>I
	1	897	A>G	ATC AAA CAG	K>R
12 Trenimon 0.5 x 10^{-8}M +3mT Staggered (b)	1	49	C>A	TTA TAC GAT	Y>stop
	5	56	G>A	GTC GCA GAG	A>T
	1	59	G>T	GCA GAG TAT	E>stop
	2	84	C>T	CAG ACC GTT	T>I
	1	87	T>C	ACC GTT TCC	V>A
	1	89	T>A	GTT TCC CGC	S>T
	1	318	C>T	GTG TCG ATG	S>L
	1	329	C>T	GAA CGA AGC	R>stop
	1	344-553	del 210 b.p.	GAA GCC ...GTC GCA	na

lac I mutations (continued)

Treatment[1]	Occurrence	Position[2]	Mutation[3]	Template Sequence Context[4]	A.A.[5] Change
12 (continued)	1	419	C>G	GAC CAG GAT	Q>E
	1	485	C>A	GAC CAG ACA	Q>K
	1	569	C>T	CAG CAA ATC	Q>stop
	1	681	G>A	GAA GGC GAC	G>D
	1	872	T>C	TGT TAT ATC	Y>H
	1	1010	A>G	AAA ACC ACC	T>A
13 Trenimon 0.5 x 10^{-8}M +3mT Concurrent (b)	10	56	G>A	GTC GCA GAG	A>T
	1	125	A>G	GCG AAA ACG	K>E
	1	143	G>T	GTG GAA GCG	E>stop
	2	374	G>T	CTC GCG CAA	A>S
	2	803	G>T	ACC GAG TCC	E>stop
	1	839-915	del 77 b.p.	GTA GTG...CTG GGG	na
	1	940	G>T	CGC TTG CTG	L>F
14 Trenimon 0.75 x10^{-8}M +3mT Staggered (b)	1	56	G>A	GTC GCA GAG	A>T
	1	885	T>C	CCG TTA ACC	L>S
	1	917	G>C	CTG GGG CAA	G>R
	1	968	A>T	GTG AAG GGC	K>stop
15 Trenimon 0.75 x10^{-8}M +3mT Concurrent (b)	10	56	G>A	GTC GCA GAG	A>T
	1	125	A>G	GCG AAA ACG	K>E
	1	150	C>A	GCG GCG ATG	A>E
	1	168	A>G	AAT TAC ATT	Y>C
	1	270	C>A	GTC GCG GCG	A>E
	1	349	T>A	GCC TAT AAA	C>stop
	1	468	T>C	TTA TTT CTT	F>S
	1	803	G>T	ACC GAG TCC	E>stop
	1	928	C>A	ACC AGC GTG	S>R

[1] Treatment groups are assigned a number (1-15). Groups from the blocked experiments with menadione and trenimon are denoted by (a) and (b), respectively.

[2] Base pair (b.p.) position in lacI gene.

[3] A>G denotes a transition from adenine to guanine. Symbols: del, deletion; +, +1 frameshift; -, -1 frameshift; ins, insertion.

[4] Mutated base(s) underlined. CpG's are in bold text. Flanking repeats are in italics.

[5] Amino acid (A.A.) change is shown, including mutations to stop codons (stop), unless non-applicable (na). Amino acid symbos used are: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V,valine; W, tryptophan; Y, tyrosine.

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