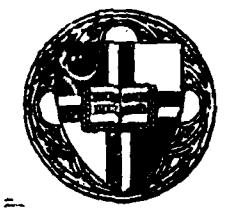

Vitreous State Laboratory

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Washington, D.C. 20064

Mechanisms of Microwave Induced Damage in Biologic Materials

Annual Report
January 1992

by

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The results of research directed at several of the very fundamental issues concerning cellular effects of weak electromagnetic fields is described. Despite their importance, these issues have not been satisfactorily addressed by the scientific community. They include the issues of (a) replication of experimental effects; (b) how weak electromagnetic fields can affect cells that exist in an electrically noisy environment; and (c) the explanation of complex dose-response relationships.

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FOREWORD

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CHAPTER 1

Overview

Research during Year 5 of this project (September 22, 1990 - September 21, 1991) was directed at tackling several of the very fundamental issues concerning cellular effects of weak electromagnetic fields. Despite their importance, they have not been satisfactorily addressed by the scientific community. They include the issues of (a) replication of experimental effects; (b) how weak electromagnetic fields can affect cells that exist in an electrically noisy environment; and (c) the explanation of complex dose-response relationships.

Our experiments unambiguously confirm the teratogenic effects of low level electromagnetic fields. The data imply that only those embryos with a genetic predisposition display a teratogenic sensitivity to electromagnetic fields. They are also definitive in establishing that exposure to ELF and AM-modulated mirowave fields causes alterations in the activity of ornithine decarboxylase in various cell lines in culture.

An intriguing discovery that offers a clue to the signal-to-noise problem is the observation that EM fields applied for durations of several hours must exhibit temporal coherence for times of the order of at least 5 seconds or so if the signal transduction mechanism is to respond and bioeffects are to occur. This provides a first-level mechanism in the cell's noise discrimination process. The hypothesis that spatial coherence of the applied field is also a requirement for field-induced bioeffects provides an exclusionary mechanism that explains the cell's ability to reject endogenous thermal fields while simultaneously responding to externally impressed signals.

We have shown that electromagnetic field exposure produces a transient enhancement of both the production and degradation rates of an intermediate reaction product (mRNA) in the sequential chain of biochemical reactions occurring within the cell. The kinetic aspects of this behavior are connected to observations of "unusual" dose-response relationships such as power and exposure windows.

CHAPTER 2

Electromagnetic-field-induced Bioeffects

2.1. INTRODUCTION

Skepticism over the possibility of weak electromagnetic fields affecting cell function exists because endogenous thermal noise fields are larger than those reported to cause effects. Four-hour exposure to an ELF field approximately doubles the specific activity of ornithine decarboxylase (ODC) in L929 cells. The results of an experiment that tests the idea that the cell discriminates against this thermal noise because it is incoherent is reported below. Although the requirement of a coherent signal was established in the experiment, the coherence times that were found to be required are of too short a duration to allow for time averaging to solve the thermal noise puzzle. A proposal that allows the cell to both respond to an exogenous field and discriminate against endogenous thermal noise fields via a cellular "coincdence-detection" scheme is outlined. In addition, studies that explore the effects of pulsed EMFs on avaian teratogenesis are described; these were undertaken to establish the existence of *in vivo* effects of ELF fields, the situation in the extant literature being apparently contradictory on this point.

2.2. BACKGROUND

The work described below continues our attempt to synthesize a framework for understanding the interaction of electromagnetic fields with cells. At its crudest level this framework pictures the interaction in terms of three major elements: (1) the direct interaction of the exogenous electromagnetic field with extracellular ionic charges just outside the cell membrane; (2) the activation of a signal transduction mechanism within the cell; and (3) the alteration of the performance of the biochemical pathways of the nucleus. Our experimental work has led to several novel results; these involve each of the items in the sequence described above.

The lack of reproducibility of results that a number of groups have documented in the literature has been interpreted in some quarters as indicating the absence of any teratogenic effect of electromagnetic fields on dveloping chick embryos. To obtain statisticaly significant results that might settle the issue, we have carried out studies of the effects of EMFs on over 3000 developing embryos. The results from exposure to pulsed EMFs are now clear--there is a robust positive effect. The EMF-induced abnormality rate seems to be inversely correlated with the abnormality rate in the sham-exposed sample. This is consistent with earlier work and suggests that a two-point genetic interaction is operative.

We have achieved major insights into one of the very fundamental questions that has long plagued the field of bioelectromagnetism: how is it possible that a cell, existing in an electromagnetically "noisy" environment, can be affected by exogenous fields that are many orders of magnitude weaker than the local fluctuating endogenous fields? We have demonstrated that if the parameters characterizing the field are caused to vary on time scales shorter than this, there are no biological effects. Even with this biochemical demand for temporal coherece, it remained a mystery as to how the weak impressed fields--still buried in the thermal noise fields around the cell--could have any effect. We now believe that our results imply that relatively weak *coherently applied signals* can alter cellular functions because they are able to affect simultaneously a large number of receptors in the cell membrane. Cooperativity among receptors is needed to cause modification of the biochemical reaction pathways. The cell discriminates against thermally generated noise fields at its surface by imposing two requirements on external fields that must be satisfied if signal transduction is to occur. These are (1) temporal coherence, that is time averaging of EM signals over times in excess of about five seconds; and (2) spatial correlation of the EM field over distances encompassing many receptors (on the order of 1 μm) so that coincident detection at several locations on the cell membrane is achieved. This interpretation of the data provides the explanation of how the noise barrier established by random ionic motion in the vicinity of the cell can be penetrated.

2.3. EFFECT OF COHERENCE TIME OF THE APPLIED MAGNETIC FIELD ON ORNITHINE DECARBOXYLASE ACTIVITY

2.3.1. INTRODUCTION

Concern over possible adverse health effects resulting from exposure to electromagnetic fields (EMF) has generated an increasing effort to determine how fields interact with biological systems. Results from cell culture studies have documented alterations in cell metabolism after exposures to extremely low frequency fields[1]. Such data make it clear that EM fields interact with cells and affect their metabolism, but, neither the mechanisms of the interaction nor the long term biological consequences of such responses are understood. Many of the reported EMF effects have been obtained with applied time varying magnetic fields as low as 1 μT with associated induced electric fields below 1 μV/cm. The magnitudes of such fields are well below the random thermal noise fields generated by the thermal motion of ions in and about the cell.[2,3] It is, thus, a mystery as to how cells can detect, and respond to them.

Because an important characteristic of thermal noise fields is their incoherence, we have explored the possibility that the cell's signal transduction mechanism might demand a certain degree of coherence in the applied fields before it would respond to them. In this way the thermal field would be ignored by the cell. We have explored this concept experimentally by asking whether, during exposure, a time varying EMF must maintain coherency over some minimum interval to elicit a cellular response. The coherence time is loosely defined as the time interval over which we can reasonably predict the frequency, phase, and amplitude of the field. The biological endpoint selected for this purpose was the EMF-induced enhancement of specific activity for the enzyme ornithine decarboxylase (ODC) in murine L929 fibroblasts. The effect of the signal coherence time was examined for 60 Hz magnetic fields.

2.3.2. METHODS

Logarithmically growing cultures of murine L929 cells, maintained in Eagle's minimum essential medium with 5% fetal bovine serum, were plated 24 hours prior to magnetic field exposure. To avoid serum stimulation of ODC activity, the culture medium was not changed before experiments were begun. ELF exposures were conducted using incubator-housed Helmholz coils to produce a sinusoidal, 60 Hz horizontal magnetic field of 10 μT. Four 25 cm^2 flasks of cells were used for each exposure and to serve as controls four identical flasks were placed in an incubator chamber adjacent to that housing the Helmholz coils. At the end of exposure cells were harvested by gentle scraping, washed with phosphate buffered saline and stored as frozen pellets. Ornithine decarboxylase activities were assayed by the procedure of Seely and Pegg[4] modified by addition of 0.2% Nonidet P-40, 50 μg/ml leupeptin, and 50 μM pyridoxal-5-phosphate to the cell lysis buffer. Results of each set of experiments are expressed as the mean ratio of the enzyme activities of exposed cultures to those of the corresponding controls (± SEM).

Coherence times of the exogenous fields were varied from 0.1 to 50 s. The coherence times were determined by a computer program which interfaced with a function generator to determine the ELF frequency and also the time interval for which a given frequency was maintained. At user-selected intervals (henceforth termed coherence times, or τ_{coh}) the frequency of the ELF field signal was alternately shifted from 55 Hz to 65 Hz (see Figure 2.3.1). The phase of successive intervals was randomized by inserting a small uncertainty in τ_{coh}. Thus the time between frequency shifts was actually $\tau_{coh} - \delta t$ where $\delta t << \tau_{coh}$ and is a random time which varied between 0 and 50 ms.

2.3.3 Results and Discussion

Cultures were subjected to a series of exposures to 60-Hz magnetic fields of 1, 10 or 1,000 μT, for times ranging from 1 to 8 hours. The enhancement of ODC activity was measured in terms of the ratio of exposed/control activity. Maximal enhancement of ODC activity (2.04 ± 0.21) was produced by 4-hour exposure to a magnetic field of 100 μT. The associated induced electric field was approximately .04 μV/cm. Comparable enhancements of ODC activity (1.79 ± 0.20, 2.10 ± 0.35) were obtained with frequencies of either 55 or 65 Hz. Using 4-hour exposures, 100 μT-fields, and frequencies shifting alternately between 55 and 65 Hz, we varied the coherence times from 0.1 to 50.0 s.

The results are plotted in Figure 2.3.2 They show that application of fields for four hours but with coherence times of 10 or 50 s did produce enhancements in ODC activities. The amount of enhancement was (within experimental accuracy) the same as that observed after exposures which were coherent for the full four hours of exposure. In contrast, for a coherence time of 1.0 s no enhancement of ODC activity was observed. A 5-s coherence time produced a level of enhancement (1.54 ± 0.06) that was intermediate between control values and those obtained with τ_{coh} of 10 s or longer.

The ratio of exposed/control ODC activity, R[ODC], plotted in Figure 2.3.2 was fit to the equation

$$R[\mathrm{ODC}] = 1 + \Delta R\,(1 - e^{-\tau_{coh}/\tau_{cell}}) \qquad (2.3.1)$$

with best fit values of $\Delta R = 1.26$ and $\tau_{cell} = 8.2 \pm 3$ s. Thus there appears to be some fundamental time constant, τ_{cell} associated with the cell signal transduction mechanism. For the cell to respond to an ELF signal it is necessary for the exogenous field to maintain coherence for a minimum time interval greater than about several seconds, with full response requiring an interval greater than about 10.0 s. Some sort of signal averaging thus appears to function in producing field-induced enhancement of ODC activity by L929 cells.

We now consider whether this coherence phenomenon will be sufficient to account for the ability of cells to discriminate against the thermal noise caused by thermal fluctuations in the position of nearby ions. To determine the thermal electrical field noise we use the Johnson-

Nyquist expression where the time average noise voltage V_{kT} and electric field E_{kT} are expressed as

$$\langle V_{kT}^2 \rangle = \frac{4\rho kT\Delta\nu}{d} \; ; \quad \text{and} \quad E_{kT} = \frac{\sqrt{\langle V_{kT}^2 \rangle}}{d}. \tag{2.3.2}$$

In these expressions ρ is the resistivity of tissue, $\Delta\nu$ is the band width of the cell signal transduction mechanism, d is the diameter of the cell, k is the Boltzmann constant, and T is the absolute temperature. Following Adair[3] we use these expressions assuming that d = 20 μm, ρ = 2 Ω·m, and $\Delta\nu$ = 100 Hz. This predicts that $E_{kT} \approx .02$ V/m. Thus we find that the thermal noise field is about 5,000 times larger than the magnitude of the 60-Hz electric fields induced in this experiment.

How does the requirement of a minimum value of τ_{coh} affect the signal-to-noise calculation? Weaver and Astumian[2] have suggested that if signal averaging is present the minimum detectable field would be given by the expression

$$E_{\min} = \frac{\Xi_{kT}}{\sqrt{\nu\tau_{avg}}}, \tag{2.3.3}$$

where ν is the frequency of the applied signal and τ_{avg} is the time over which the cell averages the signal. If we assume that that $\tau_{avg} \approx \tau_{cell}$ the minimum detectable field is still over 100 times larger than the applied fields used in this experiment. In fact, to obtain an improvement in signal-to-noise of 10,000, the averaging process would have to last for about 10^6 seconds (i.e. > 100 hours) which is clearly an unreasonable averaging requirement.

Even though our results do not explain the signal-to-noise puzzle (see Section 2.5 below), the necessity for a minimum coherence time will have to be accounted for in any model proposed for the mechanism by which cells detect an applied EM field. In addition to EMF frequency and time of exposure, coherence time must be considered an important factor in determining the cellular response.

ODC is a critical enzyme, required for DNA replication and cell proliferation, and so modification of its enhancement by an applied field is of general interest for questions of EMF exposure. We suggest, however, that the coherence phenomenon noted in these experiments is likely of more widespread consequence, and that other biological responses with demonstrated EMF sensitivity will display comparable coherence dependence. Indeed a similar effect has been observed in studies of EM induced abnormalities in chick embryos.[5]

2.3.4 FIGURES

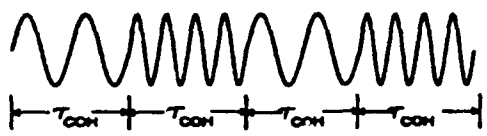

Figure 2.3.1. A plot of the partially coherent waveform created by shifting frequencies from 55 to 65 Hz at intervals of time, $\tau_{coh} \pm \delta\tau$, where $\delta\tau$ is a randomly chosen interval ($<< \tau_{coh}$) between 0 and 50 ms.

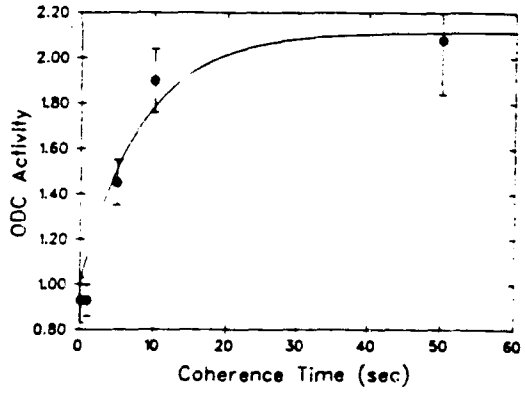

Figure 2.3.2. Plot of the enhancement of ODC activity (exposed/control) as a function of the coherence time, τ_{coh}, of the applied field. The solid line is the best fit to the mathematical function given by Eq. (2.3.1) with τ_{cell} = 8.2 s. The experimental points shown represent a minimum of six different exposures.

2.3.5. REFERENCES

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2.4. AVIAN TERATOGENESIS

Nearly a decade ago Delgado and co-workers[1] reported that low level, low frequency electromagnetic fields could seriously affect the early development of chick embryos. Although their results are at best only marginally significant from a statistical viewpoint, the idea that developing chick embryos might serve as a model system for investigating the potential health hazards of extremely low frequency (ELF) magnetic fields has stimulated much research during the intervening years. A number of subsequent avian studies have corroborated the teratogenic effects of 1 to 10 μT magnetic fields.[2,3,4,5,6,7,8]

However, other investigators have not been able to replicate this sensitivity of chick embryos to electromagnetic fields during their early development.[9,10,11,12] Consequently, considerable controversy has evolved regarding the teratogenic potential of low level magnetic fields. To resolve the issue the EPA conducted a series of parallel experiments under carefully specified conditions--the so-called "Henhouse" experiment.[13] Six different laboratories used duplicate equipment and identical exposure conditions to assess the effect of weak electromagnetic fields on early White Leghorn chick developmenmt. Despite meticulous efforts to eliminate experimental differences between the laboratories, there were significant differences in the results. Out of six laboratories, two found significant increases in abnormalities following exposure, two found a small but not statistically significant increase, and two found no effect at all.

We are left with the perplexing question of why some laboratories obtain robust results demonstrating sensitivity of the developing embryo to electromagnetic fields, yet other labs find no effect at all. Realizing that simply adding one more experimental result to the already-confued situation would certainly not of itself resolve anything, we, nevertheless, decided that only by attempting to replicate the findings would we be able to identify any previously-ignored confounding factors that might account for the inconsistencies. The resuts of these experiments, our interpretation of them, and their relevance to the earlier studies cited above are presented in the remainder of this section.

2.4.1. EXPERIMENTAL METHODS

The experimental techniques that we used duplicated those of the "Henhouse" experiment. In fact, two of our exposure systems were actually used in one of the six "Henhouse laboratories" (EPA, Maryland site). Two additional exact replicas were built. As in the Henhouse experiment VWR Model-6000 water-jacketed incubators were used; however, to avoid any stray fields possibly arising from the coiled heater element located below the water jacket at the bottom of the incubator, the water was heated externally using RTE Model-110 FRC Bath/Circulators. The incubators were used interchangeably and randomly for "exposed" and "sham" configurations.

Fertilized White Leghorn eggs were obtained from Truslow Farms of Chestertown, Maryland and were used within five days of their being layed. Just as in the "Henhouse" investigations, unipolar, pulsed, magnetic fields (500μs pulse duration, 100 pulses per second, 1μT peak intensity, with a 2 μs rise time and fall time) were employed. These pulses were

applied to the exposed eggs during the first 48 hours of incubation. Simultaneously an equal number of control eggs (10 in each run) were sham-exposed. After the 48-hour incubation, embryos were removed from their shells and examined histologically by procedures described by Berman.[14] This evaluation was performed under blind conditions. Eggs were first examined for fertility and the embryos were determined to be live or dead. Live embryos were examined for abnormal morphologies. Embryos were considered to be abnormal if they differed markedly from the Hamburger and Hamilton[15] 48-hour developmental stages. Malformations were classified as cephalic nervous system, truncal neural tube, heart, boood vessels, and somites.

2.4.2. RESULTS

Our early experiments (February-April, 1990) indicated a robust teratogenic effect; with the percentage of abnormal embryos in the control sample approximately equal to 10% of the live embryos, abnormal embryos in the exposed sample were approximately 25%. This agrees quite well with the two "Henhouse" laboratories that observed a significant increase in the rate of abnormal developments induced by electromagneticfield exposure. However, when the experiments were repeated in September-November 1990, these initial results could not be replicated. In December 1990 a new young flock was put into production; with this flock, the teratogenic sensitivity of the embryos to low level EM fields reappeared. Remarkably, also at this time, eggs from the initial flock (that had been used from February through November) began again to demonstrate a sensitivity to EM fields;

To obtain statistically meaningful results we have continued the campaign through the first nine months of 1991. A tabulation of the results for the overall campaign is presented in Table 2.4.1. The classification of the abnormalities according to types is shown in shown in Table 2.4.2. From this latter table, it appears that abnormal development of the truncal neural tube is the most important effect resulting from embryo exposure to electromagnetic fields.

2.4.3 ANALYSIS OF THE DATA

Because of the difficulty in achieving reproducibility of chick embryo teratogenesis results from one laboratory to another, the question of whether the results presented in Tables 2.4.1 and 2.4.2 could be attributable to chance is relevant. Consider for example the situation with regard to truncal neural tube abnormalities. Of a total of 1330 live embryos (control plus exposed) 214 (16.1%) exhibited this particular abnormality. If the electromagnetic field had no effect on the devlopment then one would have expected 103 (of 640) truncal neural tube abnormalities in the control group and 111 (of 690) in the exposed group. Statistical characterizations of the results are shown in Table 2.4.3. The probability of obtaining values of 86 and 128 in the control and exposed groups, respectively, purely by chance is less than about 1% ($P \lesssim 0.01$). It seems clear that the field is effecting a roughly 50% increase in the rate of abnormal truncal developments. If one uses the total number of abnormally developed embryos (rather than just the truncal abnormalities)--93/640 in the control group and 137/690 in the exposed group--a similar conclusion is reached.

Having said this, one must now confront the results of the Henhouse experiment, where, as described above, only two of six laboratories found a robust positive effect. One explanation of this difficulty is statistical--in that experiment each laboratory reported the results of control and exposed samples of roughly 100 each. In principle, one should be able to combine the results from the six laboratories to obtain a single larger, and thus statistically more meaningful, experiment. These data are shown in Table 2.4.3. The comparison of these results with our own suggests that the two data sets are consistent and strengthens the conclusion that electromagnetic fields do indeed have teratogenic consequences.

Of course, one should ask if combining the six data sets of the Henhouse experiment is a reasonable thing to do. If the project had been carried out as six identical replications of a specific experiment, then this pooling of the results would be a sensible procedure. However, for a variety of reasons, several of the participating laboratories used eggs that, in some way differed from the prescribed norm. In one case, locally supplied eggs from White Leghorn hens often exhibited an abnormality rate in excess of 50%, so eggs from a different strain of hens were used. In a second case, the eggs were obtained from a small flock especially set up for the study, creating the possibility that the normal genetic diversity that would be expected might be absent. In a third case, eggs had to be flown in from an out-of-state supplier, increasing the possibility that they were subjected to unusual (and unknown) stresses. If the data from these three laboratories is removed from the tabulation, then the ratio of the abnormal live embryo developments to the total number of live embryos is 30/271 = 0.111 in the control sample and 56/274 = 0.204 in the exposed group. While these numbers are not far from those for the other experiments (particularly in the case of the abnormal rate for exposed eggs), they are statistically less meaningful simply because of the sample size. The important point here is that whether the "controversial" results are included or not does not alter the conclusion: *exposure to a weak electromagnetic field increases the rate of teratogenesis in developing embryos.*

Although we regard this conclusion as being solidly established at this point, it is puzzling as to why the observation of a positive teratogenic effect should be so variable from one laboratory to another, and, indeed, in our own laboratory from one campaign to another. In examining our results during Fall 1990 (when we were finding no statistically meaningful electromagnetic-field-induced increase in the abnormality rate), we noted that the major difference between the early-1990 and late-1990 runs was the number of abnormalities observed in the sham-exposed control eggs. In the later runs, abnormality rates in the unexposed embryos averaged slightly over 20% compared with the earlier 10% rate. Extensive testing for stray magnetic fields in the laboratory showed no difference when compared to the ambient fields measured during the February-April 1990 period. We then discovered that other users of eggs from our suppliers were also experiencing high abnormality rates in their controls. The reappearance of field-sensitivity in December 1990 and throughout 1991 has coincided with a general trend toward lower abnormality rates in the control samples.

We therefore set out to examine the proposition that the fraction of abnormal embryos in the control group is anti-correlated with the fraction of field-induced abnormalities: the lower the rate of abnormals in the control group, the higher is the field-induced abnormality rate. In testing

this proposition, we have used several techniques for treating the data. The basic question is how to establish a "run control rate" (RCR) against which to compare a "run exposed rate" (RER). A run generally consists of simultaneously incubating approximately twenty eggs, ten of which are exposed to the pulsed EMF, with the other ten being sham-exposed.

Method 1: Moving averages

Because of the small sample involved in a single run, the number of abnormal embryos in the control group undergoes rather large fluctuations; indeed, with an average abnormality rate in the vicinity of 1 in 10, a fluctuation of ±1 corresponds to a 100% variation. To smooth out the run-to-run variations, a moving average of order 5 was used. That is, for a given run, an effective or average RCR is obtained by averaging the abnormality rates of five runs: the two prior to the run of interest, the run of interest, and the two following the run of interest. For the n^{th} run, this average, denoted by $<(RCR)_n>$, is obtained from the formula

$$<(RCR)_n> = \frac{\sum_{j=n-2}^{n+2} a_n}{\sum_{j=n-2}^{n+2} L_n},$$

where a_n is the number of abnormal embryos out of a total of L_n live embryos in the n^{th} run. We then sorted the data into four bins based on the $<(RCR)>$ values. The boundaries marking the upper and lower bounds of the bins were selected to make the number of exposed embryos in each bin as nearly the same as was possible. The results are displayed in Figure 2.4.1. The rate of field-induced abnormalities is seen to decrease with increasing abnormality rates in the control sample. For the three lower control-sample abnormality rates, this decrease is accounted for almost entirely by the increase in the control-sample abnormality rate; the percentage of abnormal embryos in the exposed samples is essentially constant. This constancy breaks down at the highest control-sample abnormality rate bin (>20%) where there is practically no difference between the exposed-sample and control-sample abnormality rates.

This result seems to confirm hypothesis that motivated the approach in the first place. To test that the systematic variation obtained in Fig. 2.4.1 is not an artifact of the particular choice of bins, the analysis was repeated using (a) three bins chosen as above, (b) five bins chosen as above, and (c) four bins chosen arbitrarily as <10%, 10%-15%, 15%-20% and >20%. For these cases, the same systematic pattern is repeated.

Method 2. Monte Carlo Assignments

A second approach involved first sorting the individual runs into groups based on the number of abnormally developed embryos (0, 1, 2 ...) in the control sample for the run. These were then sorted into RCR bins by the following procedure. For convenience we label the bins 01, 12, 23, etc. (the reason for this labeling will shortly become apparent). Each run in which

there were no abnormals in the control sample was placed in the first bin (01). If one abnormal embryo was found in the control sample, then a random number R in the interval 0 to 1 was selected and associated with that run. If $R \leq 0.5$, the run was placed in bin 01; for $R > 0.5$, in bin 12. Similarly if two abnormal embryos were found in the control sample, the same random number assignment procedure was carried out. Now for $R \leq 0.5$, the run was placed in bin 12; for $R > 0.5$, in bin 23. The process is continued for runs in which there were 3, 4 ... abnormal embryo developments in the control sample, until all runs are placed in bins. This procedure was repeated five times and the results averaged to obtain rates of abnormal developments for the control and exposed groups as a function of RCR. These results are plotted in Figure 2.4.2. As was the case in the "moving average" method, the pattern of a high rate of field-induced abnormalities when the control abnormality rate is low is seen.

2.4.4 DISCUSSION

To explore further the effect of the proportion of abnormal controls on the sensitivity of developing chick embryos to EM fields, we reviewed the published literature and compared it with our results. These data are listed in Table 2.4.4 in order of increasing proportion of abnormal controls. Ignoring temporarily the data from Umeå, Sweden (these will be considered separately below), a qualitative inverse correlation between the fractions of control and field-induced abnormals can be seen. The laboratories reporting teratogenic effects had similarly low proportions of abnormal controls (below 15%), while the laboratories reporting no effects from electromagnetic field exposure had high rates of abnormal embryos in the controls (above 20%). Figure 2.4.3 shows the correlation quite clearly. Also interesting is the observation (still ignoring the Umeå data) that the total number of abnormals in the exposed group was relatively constant from one laboratory to another, ranging from a low of 20% to a high of 30%, with all except the EPA-Las Vegas data clustering in the range 20% to 24%.

The relative constancy of the total number of abnormals in the exposed group is intriguing and highly suggestive. It points to a genetic difference in chick embryos' susceptibility to electromagnetic fields; the idea is that only susceptible embryos will respond adversely to EMF exposure. This observation is consistent with standard notions in teratology and observed differences in human susceptibility to teratogens. Even embryonic exposure to thalidomide, one of the most potent human teratogens, during the critical developmental period results in a limb abnormalities in no more than 20% of the cases.[16,17] A review of chemical human teratogens demonstrates that abnormalities occur at rates less than (and usually substantially less than) 40%. Higher abnormality rates in the teratology literature occur only when the frequency of malformations is calculated from case reports. In these instances, the inherent bias of spontaneous reporting generally leads to overestimates as normal outcomes are less reliably reported.

The observation of a "ceiling" in the abnormal development rate suggests that the teratogen alone does not determine whether an embryo will be affected; an additional genetic susceptibility factor is indicated with malformations resulting from the interaction of the genotype and environmental factors. Thus differences in teratogenesis may be considered a genetic trait,

with susceptibility or liability genes in either the maternal or fetal genotype.[18] Evidence supporting the role of genetic factors is provided by studies of the metabolism and teratogenicity of the anticonvulsant drug phenytoin. Phenytoin is metabolized to highly reactive arene oxide intermediates which are capable of covalent binding to embryonic or fetal macromolecules and nucleic acids, disrupting normal development. Individuals with low levels of epoxide hydrolase may accumulate toxic arene oxides, increasing their susceptibility to the toxic effects of epoxides. Measurement of fetal amniocyte epoxide hydrolase activity allows *prenatal* prediction of affected offspring, and provides confirmation that the teratogenicity of phenytoin is mediated, at least in part, by toxic intermediates produced during the biotransformation of phenytoin.[19,20]

A gene-teratogen interaction has also been identified with a non-chemical environmental stress. Inbred mice strains exhibit differing frequencies of heat-induced exencephaly, indicating a genetic component of susceptibility.[18] Such mice strains have also been used to demonstrate that the biological effect of an electromagnetic field can be genetically controlled--studies of the augmentation of receptor-bearing B lymphocytes by microwaves indicate that the susceptibility was controlled by a single dominant Mendelian gene.[21]

Adopting the hypothesis that EMF-induced teratogenesis requires genetic susceptibility on the part of the embryo offers the basis for ignoring the data obtained at Umeå, Sweden as part of the Project Henhouse effort. Only this laboratory did not use eggs obtained from a large commercial flock; we hypothesize that the low abnormality rates observed in this experiment reflects the fact that the small Swedish flock (25 hens compared with the order of 1000 in a commercial flock) was less genetically diverse than commercial flocks and, in this case, was characterized by a inherent susceptibility rate that is substantially lower than is obtained in a larger sample, more representative of the general population of this strain of hen.

We conclude that in the appropriate experimental model--white leghorn chick embryos--with a low incidence of genetic abnormalities, low level electromagnetic fields do indeed induce abnormalities in these embryos. Not all embryos are susceptible to EM-induced abnormalities, with the differing susceptibilities likely based on a genetic predisposition. In such a case, prior environmental stresses, such as extreme heat, may mask the field-induced effect in the experimental model.

2.4.5. TABLES

Table 2.4.1. Summary of results

	Sham-exposed embryos	Field-exposed embryos
Live embryos	640	690
Live embryos showing abnormal developments	93	137
Dead embryos	30	28
Twins	2	4
Unfertilized eggs	23	8
Broken eggs	33	28
Total number of eggs used	728	758

Table 2.4.2 Breakdown of abnormal developments in live embryos.

	Sham-exposed embryos	Field-exposed embryos
Live embryos	640	690
Live embryos showing abnormal developments	93	137
Truncal neural tube	86	128
Cephalic nervous system	51	55
Somites	6	7
Heart	3	9
Blood vessels	1	1

Table 2.4.3 Comparison of the results of this work with those of Project Henhouse.

	Project Henhouse[a]		This work	
	Control	Exposed	Control	Exposed
Live embryos	538	548	640	690
Live embryos showing abnormal developments	82	114	93	137
Percentage abnormal embryos	15.2	20.8	14.5	19.9
χ^2	5.7		6.6	
P	0.02		0.01	
"Null-result" expectation of abnormal embryos	97	99	111	119

[a] Data from Reference 13

Table 2.4.4. Comparison of the results of various laboratories.

LAB CODE	Laboratory [Reference]	Percentage live embryos with abnormalities		
		Sham	Exposed	EMF-induced
A	Univ. Western Ontario [13]	6.4	20.6	14.2
B	Umeå, Sweden [13]	8.4	12.6	4.2
C	FDA, Rockville, MD [13]	9.7	22.2	12.5
D	Univ. Kentucky/Mt. Sinai [11][a]	14.3	20.5	6.2
E	Catholic University of America	14.5	19.8	5.3
F	Madrid, Spain [13]	17.1	20.4	3.3
G	Univ. Rochester [10]	21.6	24.1	2.5
H	Univ. North Carolina, Chapel Hill, NC [13]	21.6	21.5	-0.1
I	EPA, Las Vegas, NV [13]	27.0	30.1	3.1

[a] Data are for 24-hour exposure. They are included since Martin [Ref. 7] has shown that there is no significant difference in the 24- and 48-hour-exposure results.

2.4.6. FIGURES

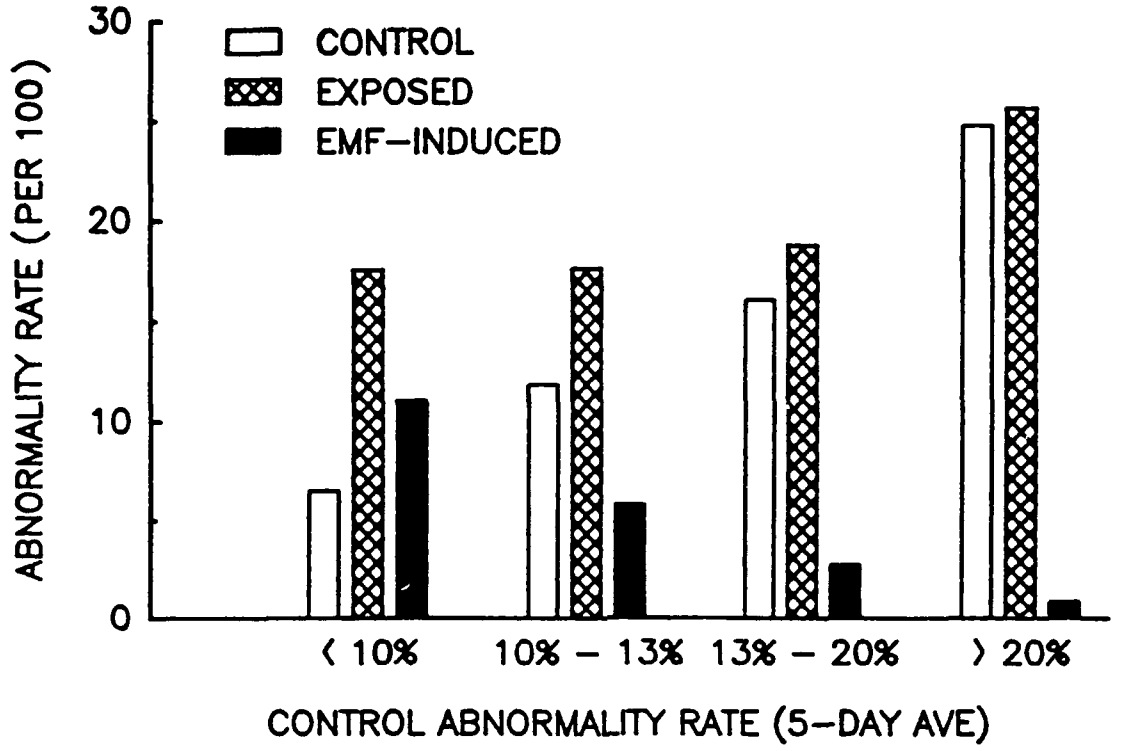

Figure 2.4.1. The meaured rate of abnormal embyo development (per 100 live embryos) plotted versus the five-day average "run-control-rate." The bars designated "EMF-induced" are obtained by subtracting the abnormality rates for the control samples from those for the exposed samples.

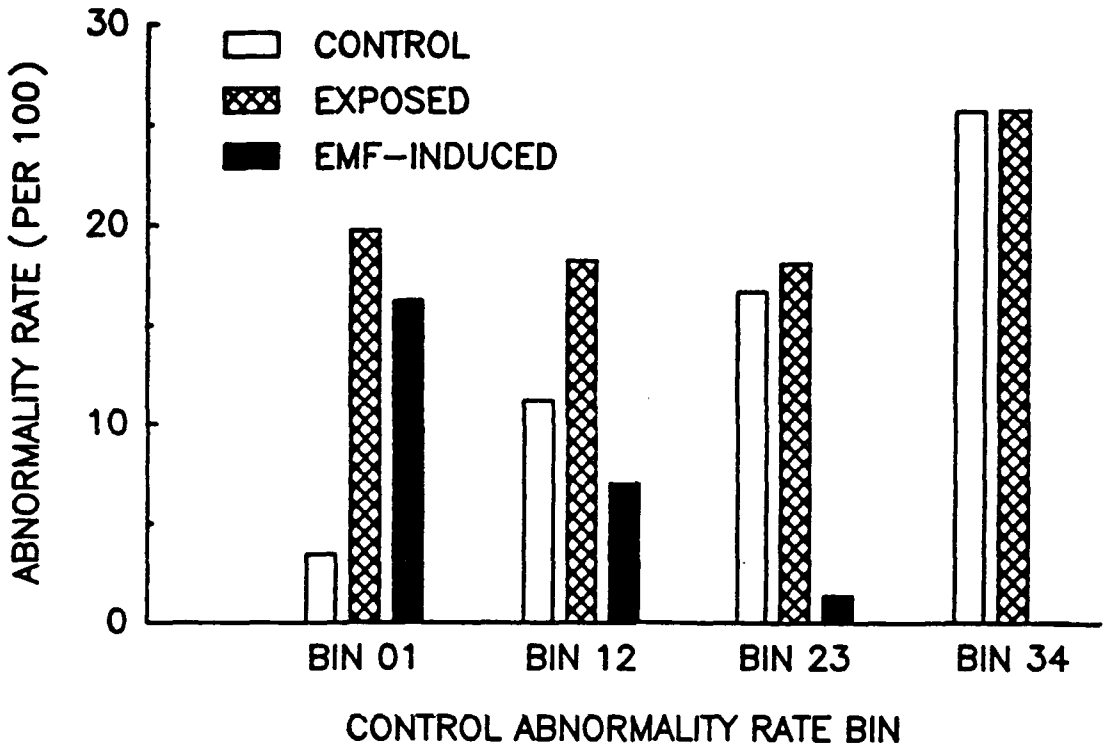

Figure 2.4.2. The meaured rate of abnormal embyo development (per 100 live embryos) sorted into bins determined by the number of abnormal embryos for the control sample for the run. The bars designated "EMF-induced" are obtained by subtracting the abnormality rates for the control samples from those for the exposed samples.

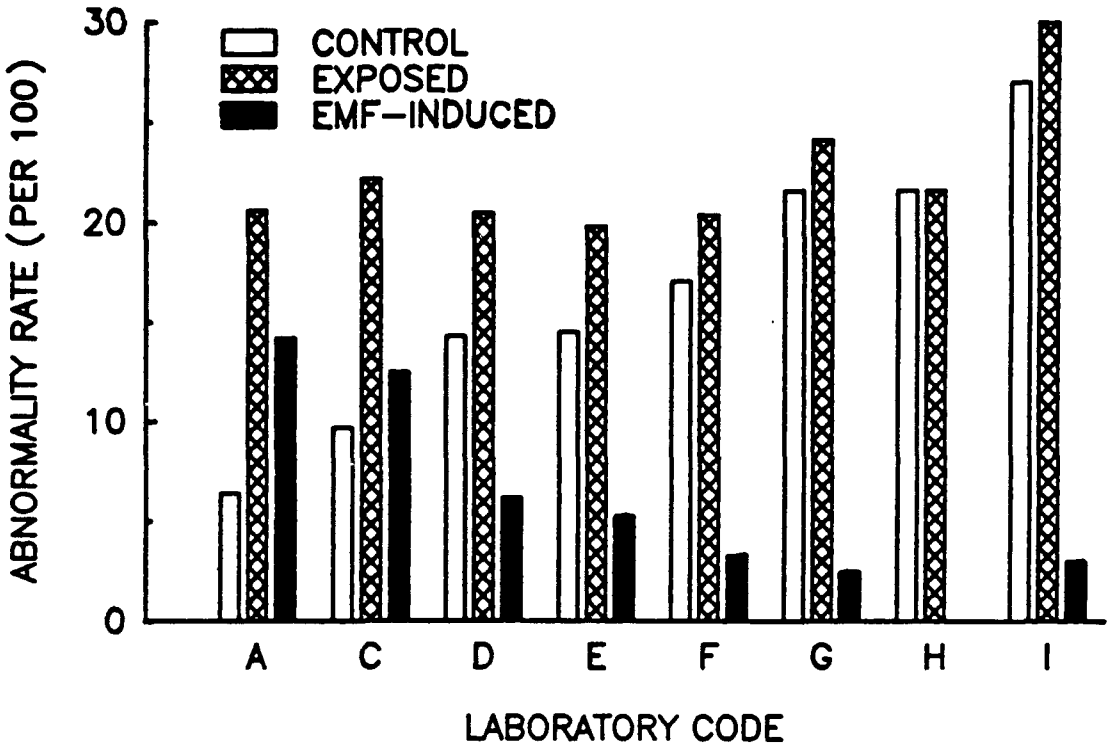

Figure 2.4.3. The meaured rates of abnormal embyo development (per 100 live embryos) for eight laboratories (the identifications are given in Table 2.4.4). The bars designated "EMF-induced" are obtained by subtracting the abnormality rates for the control samples from those for the exposed samples.

2.4.7. REFERENCES

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2.5. SIGNAL-TO-NOISE QUESTIONS: HOW CAN WEAK FIELDS AFFECT CELLS?

Cells exist in an electrically noisy environment. We propose a mechanism to account for their response to extremely-low-frequency electromagnetic fields that are considerably weaker than the thermally generated fluctuating local fields. The basic idea is that a significant number of sensors at the cell membrane must be simultaneously and coherently activated (coincidence detection) to produce an effect on the biochemical functioning of the cell. Some experimental evidence is offered to support this idea.

2.5.1 BACKGROUND

Initially stimulated by a group of less-than-conclusive epidemiological studies,[1] the issue of whether low-level extremely low frequency (ELF) electromagnetic fields can cause observable biological effects has become the focus of a scientific debate.[2] Beset by replication difficulties, early experiments often confused rather than clarified the situation. Signal-to noise questions--the field values for which effects are reported are orders of magnitude smaller than the rms value of endogenous noise fields produced by the thermal motion of charges around biological cells--are also troubling.

The replication problem, which has fueled much of the skepticism about the reality of field-induced bioeffects, can probably now be disregarded. Recently, there have been a number of independent duplications of experimental results. No fewer than four laboratories have detected an ELF-field-induced enhancement of RNA.[3,4,5,6,7,8,9] Several groups have demonstrated increased bone cell proliferation,[10,11,12] while others have detected increased activity of ornithine decarboxylase, an enzyme essential to DNA replication and cell growth, attributable to weak ELF electromagnetic field exposure.[13,14]

There are also cases in which a lack of reproducibility is now understood in terms of subtle differences in the experimental conditions. An archetypal example of this is the attempt to link altered chick embryo development with exposure to electromagnetic fields. Interest in this was generated by a 1982 report[15] that exposure to weak 100-Hz pulsed magnetic fields during the first 48 hours of incubation caused an increase in the rate of abnormal embryo developments. Although the statistical significance of the results was questionable, the work was seminal and inspired numerous repetitions of the experiment. Some of the groups obtained confirmatory data[16,17,18,19] while others were unsuccessful in finding any field-induced abnormalities.[20,21,22] To address this confusion, a six-laboratory collaboration was organized in which each participant carried out the experiment under identical conditions.[23] The result was that two of the six laboratories reported a positive effect while the others saw none (of statistical consequence).

Within the past year, order has emerged from this apparent chaos.[24] The key is recognizing that for electromagnetic fields to induce developmental abnormalities, a genetic predisposition is necessary; moreover, at least in the strain of chick embryos tested, only about

20 or 25% have this predisposition. No matter what the exposure conditions, the abnormality rate can not exceed this fraction. When (because of other than electromagnetic stresses) this value is approached in the unexposed control group, there can be *no additional* "field-induced" abnormalities. A strong negative correlation between the rates of field-induced abnormal developments and control-group abnormalities is maintained in all the data sets. In those experiments in which the control group exhibits a high abnormality rate ($\gtrsim$ 20%), no field-induced abnormalities were reported; when the control-group abnormality rate is small ($\lesssim$ 15%), a robust field-enhancement of the abnormality rate was observed. The mystery surrounding the inconsistent observations of field-induced teratogenesis has been unraveled; the data from *eight different laboratories* now form a consistent picture.

The general point is that one can no longer simply dismiss electromagnetic field-induced changes in cell functioning with the assertion that there is no credible evidence for their existence. Having said this one must now confront the second issue--how very weak ELF electromagnetic fields can affect cells that are immersed in an electrically noisy biological environment. This point has been considered recently by Adair[25] who, in this context, concludes that "... it does not appear to be possible for weak external ELF electromagnetic fields to affect biological processes significantly at the cell level ..."

Yet the data demand that they must. Examining this question is the subject of this paper.

2.5.2 The Experimental Dilemma

Briefly stated the dilemma derives from the consideration that bioeffects seem to be produced with impressed 60-Hz magnetic fields of less than about 1 μT corresponding to induced electric fields typically of 10 μV/m strength. At the same time thermal fluctuations lead to local movements of ionic charges and thus electric "noise" fields with rms values some 100 to 10,000 times larger than this.[25] The question is, of course, how the former can possibly influence cell behavior when the cell has evolved in such a way as to function normally in the presence of the latter. Or phrased another way, how does the cell discriminate against the large thermal noise fields in order to respond to the weak exogenous fields?

Weaver and Astumian[26] have proposed that cells integrate the electromagnetic signals and thereby effectively narrow their acceptance bandwidth, thus averaging out the thermal noise. They estimate that to achieve the required signal-to-noise improvement at 100 Hz requires averaging over 4.3×10^4 s (about 12 h); yet there are many cases of bioeffects being observed with exposure intervals substantially less than one hour. It is clear that a simple time averaging mechanism cannot explain the data.

Litovitz, Mullins and Krause[27] have suggested that an important element in the cell's discriminating against thermal noise involves the temporal coherence in the exogenous field (see Section 2.3 above). Their finding that some type of time correlation for about 10 s is essential in the field-induced enhancement of both ODC activity in L929 cells and abnormal development

of chick embryos suggests that cells must carry on a kind of signal processing that allows them to disregard fields that are incoherent on time scales shorter than this. It is also clear that this is not the whole story. Ten seconds of signal averaging is not sufficient for detecting a 60-Hz signal that is a factor of 1000 weaker than noise. Moreover, the field at any point is the sum of the exogenous field and the fluctuating noise field. Since there can be no question that this field exhibits temporal incoherence on a scale much less than 10 seconds, the question remains--how does the cell discriminate against the noise field in order to respond to the exogenous field?

2.5.3 Biological Cooperativity: A Coincidence-Detection Mechanism

A clue to the mechanism of noise field discrimination is provided by the chick embryo results of Juutilainen and his co-workers[28] (for 50-Hz sinusoidal electromagnetic fields) and confirmed in our laboratory that there exists a rather sharp threshold as a function of field strength for the onset of teratogenic effects. When sharp thresholds involving effects produced by chemical agents have been observed at the cellular level, biologists have interpreted the data in terms of the *principle of biological cooperativity*: more than one of the cell's sensors must be simultaneously activated to induce a response. The bioresponse R as a function of concentration c is described by an expression of the form[29]

$$R \equiv (M_{exp} - M_{cont})/M_{cont} = c^n/(K + c^n), \qquad (2.5.1)$$

where M denotes the actual property being monitored, the subscripts *exp* and *cont* refer to the experimental and control systems, K is an equilibrium constant, and n is the Hill coefficient, approximately the number of sensors that must be simultaneously activated. This idea can be adapted to the case of response to an ELF field. For example, one can assume that the presence of the field affects the binding of ligands to receptors (sensors) at the cell membrane, thereby altering the probability of their activation. Then

$$R = E^n/(A + E^n), \qquad (2.5.2)$$

where E is the field strength and A is a constant.

This idea that a multiplicity of cell signal receptors are simultaneously activated suggests that a mechanism analogous to *coincidence detection* could be operative in discriminating against fields from thermal fluctuations. We assume that the direct "target" of the electromagnetic field is the assembly of neutralizing "counter-ions" in the immediate vicinity of the cell surface, and that the resulting motion of these ions produces effects at the membrane that are transmitted to the cell interior where modification of the biochemical reaction pathway is effected. A plausible supposition is that the ionic motion affects the binding of ligands to the roughly 100,000 receptor proteins (sensors) that are integral to the cell membrane. Binding ligands causes the production of intracellular effector molecules (second messengers) within the cell; the net effect is the transducing of the extracellular signal into an intracellular one. Cooperativity is required in such processes in that "more than one intracellular effector molecule must [simultaneously] bind to some target macromolecule in order to induce a response."[30]

Because the average spacing between receptors is on the order of 100 nm and the Debye screening length (roughly the range over which a given ion is not shielded from other ions) is about 1 nm, localized charge density fluctuations in the neighborhood of a given receptor will not influence motion of charges near other receptor proteins. Thermal noise fields thus are prevented from producing intracellular effects. Conversely, impressed ELF fields are *spatially* coherent over the cell surface and therefore produce charge density variations that are correlated at various receptor sites in the membrane. Consequently, they produce the required number of effector molecules to initiate a cytoplasmic response. This biological coincidence detection scheme allows the cell to be exquisitely sensitive to very weak *spatially correlated* electromagnetic fields while discriminating against the much stronger but spatially random (on the relevant distance scale) thermal noise fields.

To test the idea that the cell is able to recognize only spatially coherent fields, the following experiment was performed. On the 60-Hz ELF field that caused a significant increase in abnormal chick embryo development, a random signal (essentially "white" noise up to a few kHz) was superimposed. This sinusoid-plus-noise field was applied to the incubating embryos. The rms value of the noise signal was set at approximately twice the value of the 60-Hz field strength (this value is much less than the thermal noise fields that are apparently innocuous insofar as affecting development during incubation). Note that while it is temporally incoherent, this externally applied noise field is spatially coherent. Based on what was said above, the biological system should be unable to discriminate against the noise and consequently, the field should have no effect on embryo development.

As anticipated no field-induced abnormalities were observed with the white noise signal superimposed on the 60-Hz field. The system is unable to distinguish the coherent signal in the presence of the noise. With the mechanism of coincidence detection denied it as a discrimination technique, the biological organism has no means of identifying and responding to the weak ELF field. This result is entirely consistent with the previously reported result in which introducing partial incoherence into the imposed field interdicted any effect of the field. Biological systems are subject to the usual signal-to-noise constraints of conventional physics; however, they seem to have evolved a rather sophisticated coincidence detection scheme for discriminating against spatially incoherent noise signals.

In summary, we have presented a hypothesis that is consistent with the extant data that accounts for the sensitivity of cells to external electromagnetic fields that are several orders of magnitude weaker than endogenous thermally-driven noise fields. The idea is that cellular response to a field requires simultaneous activation of several membrane sensors, thereby enabling cells to discriminate against spatially incoherent thermal noise while maintaining sensitivity to correlated external signals. When a temporally random, but spatially correlated noise signal is combined with a sinusoidal field, the cell's coincidence-detection discrimination mechanism becomes inoperative, it faces the usual signal-to-noise problems, and the biological effect is suppressed.

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CHAPTER 3

Cellular Effects of Electromagnetic Fields

3.1. INTRODUCTION

Research efforts of the biology group have focused on four principal areas of interest: (1) enhancement of ornithine decarboxylase activity of cultured cells as the result of electromagnetic field exposure has been further investigated, and field parameters important to cellular response have been examined; (2) field-induced alterations of steady state levels of mRNA for the *c-myc* oncogene in Daudi human lymphoma cells have been studied; (3) studies have been made of changes in rate of transcription for the *c-myc* and β-actin genes in the HL-60 and Daudi human cell lines, and; (4) work to develop a DNA library for identification of genes activated by electromagnetic field exposure has been initiated.

3.2. ELECTROMAGNETIC FIELD EXPOSURE PARAMETERS AND ENHANCEMENT OF ORNITHINE DECARBOXYLASE ACTIVITY

We have found that exposure of the murine cell line L929 to either sinusoidally varying, 60-Hz magnetic fields of 10 μT, or to 60-Hz amplitude modulated 915 MHz microwaves at an SAR of 3 mW/g results in an approximate two-fold enhancement of ornithine decarboxylase (ODC) specific activity. These results are of significant interest for two reasons. First, the data represented a clear biological response to application of a small magnetic field, and provided confirmation of field-induced ODC enhancement reported by others (Byus *et al*, 1987, 1988). Second, ODC provides the rate-limiting step in the formation of polyamines, which are essential for DNA replication and cell proliferation (Heby and Persson, 1988; Pegg, 1990). The enzyme altered by the applied electromagnetic field is, thus, one of considerable biological importance.

Work during the current year has emphasized examination of the exposure parameters which influence the extent of ODC activity in response to extremely low frequency (ELF) magnetic and amplitude-modulated microwave fields. Results of this work are presented below. ODC is a highly inducible enzyme, the activity of which can be influenced by a number of ligands or hormones binding to specific membrane receptors (Heby and Persson, 1988; Pegg, 1990). Additional research will be required to determine the signal transduction pathways through which applied electromagnetic fields actually alter cellular and molecular function.

3.2.1. METHODS

Cell Culture. Monolayer cultures of L929 cells (American Type Culture Collection CCL1) were maintained in Eagle's Minimal Essential Medium supplemented with 5% donor calf serum. No antibiotics were added to the medium. For exposures, cultures were prepared the day prior to

use, and were seeded so as to be in mid-logarithmic growth by onset of the experiment. Cultures of the human leukemic cell line HL-60 were maintained in the in the same medium, supplemented with 10% fetal bovine serum and sodium pyruvate.

Microwave Exposures. Unless other wise stated, microwave conditions were either continuous wave or 23% amplitude modulation of the 915 MHz signal. Amplitude modulation was sinusoidally varied at frequencies of 6, 16, 55, 60, 65 or 600 Hz. Specific absorption rate (SAR) for these experiments was 3 mW/g. This level of microwave exposure had previously been determined to produce no appreciable heating (less than 0.1° C rise) of the culture medium. Four 25 cm^2 flasks of L929 cells were placed into a Crawford Cell for exposure. The Crawford Cell was maintained in a 37° C cell culture incubator to provide for temperature regulation. Four control flasks were placed in the same incubator chamber, but on a shelf outside the Crawford Cell, positioned so that the two sets of culturess were at the same height.

ELF Exposures. ELF exposures were conducted using a Helmholz coil system. The coils were positioned vertically, providing a horizontal magnetic field (parallel to the cells' growth surface) and, thus, yielding a relatively uniform induced electric field across the growth surface. Exposures were conducted from 1 to 8 hr, using 60-Hz, sinusoidally varying magnetic fields of 10, 100 or 1000 μT. Cells were harvested immediately after exposure, washed, and stored as frozen pellets until processing for ODC activity.

Coherence Time. Coherence time (τ_{coh}), for exposures in which this paramter was employed, was determined by a computer program provided by the electrical engineering contingent, and which they describe elsewhere in this report. Basically, the computer program, interfaced through a function generator to the power supply, was used to determine intervals over which exposure parameters were switched. In some cases frequency of amplitude modulation of a microwave field, or the frequency of an ELF field were changed with each switching event. In other instances the amplitude of an ELF field was altered. Intervals of τ_{coh} employed varied from 0.1 to 50.0 sec.

ODC Assay. ODC activity was determined by a modification of the method of Seely and Pegg (1983). Briefly, the frozen cell pellets were lysed with 100 μl lysis buffer (25 mM Tris-HCl, pH 7.5; 2.5 mM dithiothreitol; 100 μM EDTA; 50 μM pyridoxal 5'-phosphate; 50 μg/ml leupeptin; 0.2% NP-40), vortexed vigorously for 20 sec, and centrifuged at 10,000 x g for 15 min at 4° C to yield S10 supernatants. Protein concentrations were determined on the S-10 fractions. 250 μl reaction mixtures (400 μM L-ornithine; 0.125 μCi L-[1 C] ornithine; 40 μM pyridoxal 5'-phosphate; 1.25 mM dithiothreitol; 30 mM Tris-HCl, ph 7.5) were prepared for each sample, containing 500 μg of S-10 protein added last to initiate the reaction. The reaction was carried out in conical 15 ml glass tubes sealed with rubber stoppers carrying polypropylene wells (Kontes) containing 200 μl, 1.0 N NaOH. Incubations were for 1 h at 37° C in a shaking water bath. The reactions were terminated by the injection of 300 μl 20% TCA, and a further 15 min 37° C incubation driving the generated CO_2 into the NaOH. The NaOH was pipetted from the wells into scintillation vials containing 6.0 ml Opti-Fluor scintillation cocktail, and the counts per

minute were determined with a scintillation spectrometer. Acid-killed enzyme was used to determine background. Activity units were nM ^{14}C-CO_2 generated/30 min/g protein.

3.2.2. Coherence Time and the Enhancement of Cellular ODC Activity

We previously demonstrated a relationship between the coherence of an applied electromagnetic field and the ability of a cell culture to respond to the field by displaying enhanced ODC activity. This work was initiated in response to the hypothesis of Weaver and Astumian (1990) that the ability of cells to respond to electromagnetic fields with amplitudes below those calculated for random thermal noise might depend on some sort of signal averaging over time. Accordingly, we attempted to disrupt coherence of the signal, asking if there were some minimum time interval over which the cell must perceive a coherent signal in order to detect and respond to it. Coherency disruption of an applied microwave field was done by switching the frequency of modulation of the 915 MHz signal from 55 to 65 Hz at user specified intervals termed coherence times (τ_{coh}). Shifts in frequency from 55 to 65 Hz were similarly conducted for cells exposed to 10 μT, sinusoidally varying magnetic fields. In order to assure incoherency of the signal from one τ_{coh} to another, a randomly determined interval, varying from 0 to 50 msec, was subtracted from each τ_{coh}. Thus, if a τ_{coh} of 1.0 sec were selected, the frequency would alternate between 55 and 65 Hz at 1.0 sec intervals, with each interval being shortened, randomly by 0 to 50 msec. Cell cultures were exposed for 8 hr to the microwave field, or for 4 hr to the ELF field; these exposure times had previously demonstrated maximum enhancement of ODC activity using continuously applied fields.

Results. We described in the previous annual report a τ_{coh}-dependent variation in enhancement of ODC activity for amplitude modulated 915 MHz microwaves (Krause *et al*, manuscript submitted). A τ_{coh} of 1.0 sec or less produced no enhancement in ODC activity compared to controls, but a τ_{coh} of 10.0 sec or longer produced the typical two-fold enhancement observed with a continuous exposure to a field with unvaried 55, 60 or 65 Hz amplitude modulation frequency. Intermediate values of ODC enhancement (1.537 ± 0.058) were obtained with τ_{coh} of 5.0 sec. The ability of L929 cells to respond to an amplitude modulated microwave signal thus was found to be limited by τ_{coh}.

Experiments to determine the τ_{coh} response of L929 cultures exposed to ELF fields (Litovitz *et al*, 1991) yielded results that were remarkably similar to those obtained with an amplitude-modulated microwave signal. ODC enhancement depended clearly upon the τ_{coh} utilized over the 4 hr exposure period. Full, or nearly full, enhancement of ODC activity was obtained when τ_{coh}s of 10.0 or 50.0 sec were employed; no enhancement was produced with τ_{coh}s of 1.0 sec or lower, and 5.0 sec yielded intermediate enhancement. The τ_{coh} responses for both the amplitude-modulated microwave and ELF conditions are compared in Table 3.7.1, and are graphed in Figure 3.1. The ODC enhancements in either system were quite similar for the same τ_{coh}. Signal coherence is, thus, a critical factor in determining ODC response when cells are exposed to either amplitude-modulated microwave or to ELF magnetic fields.

3.2.3. Importance of the Random Interval Modification of Coherence Time for Determining Cellular Response

Initial work with coherence time, in both the AM microwave and ELF systems, employed modification of each τ_{coh} interval to assure that from any one time point, the phase of the field could not be predicted for any subsequent time point. The random interval modification thus assured incoherence of the applied fields. Given the importance of coherence time in determining the ability of cells to respond to electromagnetic field exposure, it was necessary to determine whether the degree of incoherency provided by the nonrandom time interval was, in fact, necessary. Accordingly 1.0 and 10 sec τ_{coh} exposures were repeated for the AM microwave system, and 1.0 sec τ_{coh} exposures repeated for the ELF system, but with no random modification of the τ_{coh} intervals.

Results. ODC activities for cultures exposed to the frequency-varied AM microwave field in which the τ_{coh} was 10.0 sec were 1.943 (± 0.168) times the activities of matched control cultures. For a τ_{coh} of 1.0 sec, however, ODC activities were 1.15 (±0.280) those of matched controls. These results are comparable to those obtained with exposures in which the τ_{coh} intervals were randomly modified. The data demonstrate that the degree of field incoherence produced by the random time element is not essential in determining the cell's time-dependent sensitivity to variation in the ELF amplitude modulation frequency of a microwave field.

These results were confirmed for ELF manetic fields by exposing cultures to a field in which frequency was shifted from 55 to 65 Hz at a τ_{coh} of 1.0 sec without employment of the random time element. As expected from the AM microwave results, ODC activities were found to be comparable to those of control cultures (0.935 ± 0.156). Since ODC enhancement was eliminated by the 1.0 sec τ_{coh} whether or not the random time element was used, the phase randomization was not necessary for the effect.

3.2.4. Coherence Time Intervals and Amplitude Variation of an ELF Field

Coherence time work to this point employed changes in frequency at τ_{coh} intervals. In accordance with the fact that a frequency change also represents a shift in amplitude of the applied field, the physicists in our group suggested that experiments be done in which amplitude of the magnetic field, rather than frequency, be modified. Accordingly, experiments were conducted in which the amplitude of the applied magnetic field was changed, but frequency was maintained at 60 Hz. Thus, instead of a constant amplitude of 10 μT, the amplitude was alternated between 5 and 10 μT at intervals of 1.0 or 10.0 sec. This variation produced a field which had a mean amplitude of 10 μT, previously shown to be effective in enhancement of ODC activity. Since the nonrandom time interval had been shown not to be essential (Section I.B., above) in the frequency shift experiments, it was not employed for the amplitude variation exposures.

Results. Exposures yielded ODC activities of 1.82 ± 0.21 for τ_{coh} of 10.0 sec, demonstrating enhancement of ODC activity equivalent to that obtained when the frequency of the applied magnetic field was alternated between 55 and 65 Hz. Such shifts in amplitude, over a 10.0 sec interval, did not diminish the typical enhancement of ODC activity obtained with a constant 10 μT amplitude. Amplitude variation at a τ_{coh} of 1.0 sec, however, produced no enhancement of ODC activity in exposed cultures (0.96 ± 0.161), equivalent to the situation obtained with the same time interval and frequency variation.

These data suggest, but do not prove, that the major effects of frequency variation at τ_{coh} intervals may actually result from variations in amplitude of the applied magnetic field rather than the actual frequency variations. Certainly amplitude variation is equally effective, and displays the same time constants, as frequency variation, and thus is a factor in determining cellular response to the field.

3.2.5. Cellular Response to an Applied Field which is Alternately Switched On and Off During the Entire Period of Exposure

Given the importance of the τ_{coh} interval in determining cellular response to an applied field, a series of exposures were undertaken to determine whether a repeated ON/OFF application of a 60-Hz magnetic field could influence ODC activity levels, and whether the interval at which the field were turned off and on would be critical for determining response. In each experiment exposure was conducted for a total of 4 hr, the time previously determined to produce maximum ODC enhancement. Exposure conditions chosen were either 10 μT, switched off and on at 1.0 or 10.0 sec intervals, or 20 μT, switched at the same intervals. The 20 μT amplitude was selected to yield an average of 10 μT over the 4 hr exposure time, thus equalling, on average, the field which had demonstrated consistent ODC enhancement. An additional set of exposures were done in which the field was switched on for 0.2 sec, and then switched off for 0.8 sec; an amplitude of 10 μT was used for this series.

Results of these exposures are displayed in Figure 3.2. ODC values for the 10 and 20 μT results are comparable. Switching the field at intervals of 1.0 sec produced no enhancement of ODC activity over control cultures for either amplitude (1.09 ± 0.17 and 1.04 ± 0.16, respectively). Switching the field at 10.0 sec intervals, however, yielded ODC enhancements. Exposed cultures yielded ODC activity ratios of 1.89 ± 0.21 (10 μT) and 1.62 ± 0.19 (20 μT). The lower figure for the 20 μT group may reflect the earlier time course of enhancement displayed with increased amplitude of the applied magnetic field (see the following section, I.E.). The final experiment involving switching the field on for 0.2 sec and off for 0.8 sec during the 4 hr exposure period produced no enhancement of ODC activity (1.105 ± 0.19).

For results obtained to date the situation of switching a field off and on at set intervals corresponds to the τ_{coh} intervals employed for frequency and amplitude variation. An interval of 1.0 sec produced no cellular response to the field, but a response was obtained with an interval of 10.0 sec. The ability of the cell to respond to an applied field is, thus, consistent whether a

particular characteristic of the field (frequency or amplitude) is altered at a set interval, or the field is simply swtiched off and on.

3.2.6. Relationship Between Amplitude of the Applied ELF Magnetic Field and Ornithine Decarboxylase Response

We have demonstrated a two-fold enhancement in ODC specific activity that results from a 4 hr exposure of L929 cultures to a 10 μT, 60-Hz magnetic field. In order to understand the nature of this alteration in ODC activity, we needed to know whether enhanced enzyme activity was maintained in the cells for any significant length of time, and whether the magnitude of the applied magnetic field influenced the level of enhancement and its duration. Accordingly a series of time course exposures were run, in which cultures were continuously exposed for periods ranging from 1 to 8 hr. Three magnetic fields, differing by two orders of magnitude were utilized. Ten μT fields were employed, since an effect had already been demonstrated with fields of this intensity. Fields of 100 and 1 μT were used to provide significant brackets of intensity on either side of the field of demonstrated effectiveness.

Results. The time course for the 10 μT magnetic field, already shown to produce an approximate doubling of ODC activity after a 4 hr exposure, demonstrated the ODC response to be transient (Figure 3.3A). Exposed cultures demonstrated a lowering of ODC activity relative to controls at 2 h of exposure, but displayed some increase at 3 h and approximately 1.85 x control values at 4 h. By 6 h, ODC activity was near control values, and remained so to 8 h of exposure. The 100 μT field, although 10-fold higher, did not yield a significant difference in the magnitude of ODC enhancement (Figure 3.3B). ODC activity peaked at 3 h, with exposed cells displaying 1.95 x the specific activity of unexposed controls. Interestingly, however, although the magnitude of the response did not change with the higher amplitude field, the timing of the response did, with the values for the 2 and 3 hr time points corresponding to those obtained at 3 and 4 hr with a 10 μT field. Since the ODC activity remained high at hr 4 in the 100 μT exposed cultures, it may be that duration of ODC enhancement is also increased with the higher field. In order to determine this we have initiated a series of exposures for the 5 hr time point, but these data are not yet available.

Exposures with the 1 μT field, by contrast, produced little enhancement of ODC activity. Results of this time course are shown in Figure 3.3C. ODC activities did not differ significantly from controls at any point in the exposure series with the exception of 6 h. At this time point a 1.3-fold enhancement of ODC activities was observed, but considerable variation was obtained around this mean. There may, thus, be a slight peak of enhanced ODC activity at 6 h, but the statistics are not yet adequate to say this with certainty. To answer this question additional exposures are being conducted for the 6 hr time point, and also for the 5 hr time point, which would allow clarification of the kinetics of a minimal ODC response.

3.2.7. TIME OF EXPOSURE REQUIRED TO STIMULATE FULL ODC ENHANCEMENT

We have observed transient enhancements of ODC activity that peak at 8 hr of continuous exposure to 60-Hz amplitude modulated 915 MHz microwaves, and at 4 hr of continuous exposure to a 10 μT, 60-Hz magnetic field. The timing of the response to the 60-Hz field was particularly interesting, since it follows the same time course displayed for enhanced ODC activity by cells which are stimulated by growth factors (Hovis *et al*, 1986). The binding of ligands to specific membrane receptors triggers responses in cells through second messenger channels, often with the activation of protein kinase C or adenylate cyclase (Butler *et al*, 1991). In many cases a ligand need not remain in the culture medium, but rather a brief exposure of the cells to it is sufficient to produce a complete response minutes to hours later. Given the similarity in kinetics of the field-induced response to those observed with growth factors, we decided to examine the question as to whether some relatively minimal, initial application of the field would be capable of eliciting the same ODC enhancement as does continuous exposure.

To perform these experiments exposures were initiated at time zero, and the cells were exposed to the 60-Hz, 10 μT field for a predetermined time from 0.25 to 4.0 hr. All cultures were harvested at 4 hr from the onset of exposure, to provide the full 4 hr time interval associated with the peak ODC enhancement observed for continuous exposure. Similarly, parallel exposures were run with the microwave system, with cells exposed for periods ranging from 1 hr to 8 hr, and all cultures harvested for assay of ODC at the 8 hr time point associated with peak ODC enhancement in the microwave system. Results from the microwave series are not yet sufficiently complete for analysis, but the ELF data show an interesting trend and are presented here.

Results. Results of this "limited exposure" series are displayed graphically in Figure 3.4. The extent of ODC enhancement was found to vary with the initial exposure interval; further, a full, aproximately 2-fold enhancement did not require exposure for the full four hr. Exposure times of 0.25 or 0.50 hr yielded no significant enhancement in ODC activities over control cultures when assayed at 4 hr from onset of exposure. A 1 hr exposure, however, produced ODC activities of approximately 1.7 x control values at 4 hr from exposure onset, and exposures of 2 or 3 hrs yielded ODC enhancements at 4 hrs that were statistically indistinguishable from that obtained with a full 4 hr exposure. These data indicate that a field need not be continuously maintained in order to elicit, subsequently, a full biological response. Magnetic field exposure, hence, does affect ODC activity in a time-dependent manner reminiscent of that obtained with exposure of cells to growth factors.

3.2.8 DISCUSSION

The ornithine decarboxylase marker used to assess cellular response to electromagnetic fields in these studies is an important factor in cellular metabolism (Heby and Persson, 1988; Pegg, 1990). Without the production of polyamines, initiated by formation of putrescein from ornithine by ODC, DNA replication and cellular proliferation cannot occur. Further, ODC activity appears to have important roles in cellular differentiation and other metabolic events.

An electromagnetic field effect upon this enzyme is, consequently, of considerable interest. None the less, field induced effects are likely to be of importance for many enzymes of consequence (for example, see Byus *et al*, 1984). The chief importance of the studies reported here may be less the fact that ODC activity displays response to a field, but rather the use of ODC to delineate the physical and biological parameters involved in that response.

Our work has demonstrated ODC enhancement that relates to frequencies in the ELF region. Continuous wave microwave signals failed to elicit enhanced enzyme activity, but amplitude modulation at 55, 60 or 65 Hz produced response. It is the ELF component that modified cellular activity. The actual frequency range over which ODC activity can be stimulated remains to be determined. None the less, the responses to 60-Hz AM microwave and 60-Hz magnetic fields is basically similar. The response is transient, and involves an approximate doubling of ODC activity. Further, τ_{coh} responses of for the two exposure systems are basically identical. The major difference in response between the AM microwave and ELF exposures is in timing, with the microwave system producing full response at about 8 hr, and the ELF system at about 4 hr of exposure.

ODC enhancement via 60-Hz magnetic field exposure displays a time course similar to that seen in cultured cells treated with growth factors (Hovis *et al*, 1986). Growth factors, through signal transduction mechanisms, produce transiently elevated ODC activities that peak at 3 to 4 hrs and subsequently decline. This similarity in timing, coupled with the fact that briefer (approximately 1 hr) initial exposures to the applied field are sufficient to produce the full effect at 4 hr post exposure onset, argue for similar pathways for field-induced and ligand-induced ODC enhancements. It is reasonable to hypothesize that field-induced alterations in ODC are also acting at the level of membrane receptors, producing alterations in signal transduction pathways within the cell. Since ODC can be influenced by more than one signal transduction pathway (Heby and Persson, 1988; Pegg, 1990), it is impossible at this point to predict which receptor-pathway systems might be altered by field exposure.

Since it is unlikely that a particular membrane receptor is exquisitely sensitive to extremely weak electromagnetic fields, a more reasonable guess is that the field may affect the interaction of one or more ligands with membrane receptors, thus altering the normal course of events in signal transcuction. If this were so, then cells transferred to serum free medium prior to exposure might display a very limited enhancement of ODC since ligands would be unavailable to bind to membrane receptors. Alternative experiments would involve use of specific protein kinase inhibitors to block signal transduction pathways, or the elimination of protein kinase C from cells by prolonged exposure to phorbol ester, so as to eliminate a major pathway of signal transduction. Such experiments are planned and will be carried out as other work is completed.

A remarkable fact established by this work is the demonstration of coherence time as a significant factor in determining whether cellular response to a field will occur. All our work to date indicates a consistent set of intervals for response. Switching of amplitude modulation frequency, frequency, or magnetic field amplitude does not prevent cellular response provided

that the switching intervals are approximately 10.0 sec or longer. Intervals of approximately 1.0 sec or shorter are insufficient to allow cellular response, and exposed cultures so exposed display the ODC activities of control cells. This response is, to some extent graded, since coherence times of 5.0 sec provided an intermediate level of ODC enhancement. Experiments in which a 60-Hz field was switched off and on at 1.0 or 10.0 sec intervals also conformed to these interval requirements, indicating the general nature of the coherence time phenomenon. Members of our group have explored mathematically the hypothesis of signal averaging over time, put forth by Weaver and Astumian (1990), to see if coherence time intervals might be used to account for cellular response to weak fields (Adair, 1991). The results, discussed in their section of the report, show that coherence time alone cannot provide the answer to weak field response. None the less, it is clear that maintenance of a uniform field over some minimum time interval is essential for cells to detect and/or respond to an electromagnetic field.

The time course of ODC response to an applied magnetic field appears to vary with the amplitude of the field. In our experiments ODC activity peaked an hour earlier when the cells were exposed to a 100 μT field vs a 10 μT field. Interestingly, the magnitude of the response, an approximate doubling of ODC activity, was virtually the same for both fields. Results with 1 μT fields suggested a weak (approximately 1.3 x control values) and delayed enhancement of ODC activity, but results are not yet statistically convincing. There is, presumably, a range of amplitudes over which ODC response will vary directly, with the response maximizing at or before 10 μT. With increasingly higher amplitudes the main response may then be a shift in the time of maximized enzyme activity. We will explore this idea further by analyzing additional time courses at magnetic field amplitudes between 1 and 10 μT, and above 100 μT. It will be of particular interest to see whether combinations of field amplitudes and exposure times can be determined which will produce prolonged elevation of ODC activity, rather than the transient response thus far observed with continued exposure. Since individuals exposed to environmental fields would be expected to experience variations in field intensities and exposure times, such data may be more meaningful to human exposure under most conditions.

3.3 ELECTROMAGNETIC FIELD ENHANCEMENT OF *C-MYC* STEADY STATE mRNA LEVELS IN DAUDI CELLS

We have previously examined the question of altered transcription produced by electromagnetic field exposure. This work was done using the human promeylocytic leukemia line HL-60. Results from those studies showed that a transient, approximately 50% enhancement of total RNA synthesis resulted from exposure to magnetic fields in the 1 to 10 G range, but that no enhancement of particular mRNAs could be detected (Greene *et al*, 1991). Further analysis, using gel electrophoresis, demonstrated that the measured increase in transcription was in the 45S pre-RNA molecule, from which the 18S and 28S rRNAs are produced (Greene *et al*, submitted).

Work reported in the literature documents changes in the steady state levels of specific mRNAs as early as 20 min following exposure of HL-60 cultures to induced, extremely low frequency, electric fields in the μV/cm range (Goodman *et al*, 1989, 1991; Wei *et al*, 1990). Such increases have been reported for *c-myc*, *fos*, histone H2B and actin mRNAs. Until recently,

of the actively transcribed genes examined, all displayed similar enhancements of steady state levels; the β_2-microglobulin gene, however, has now been reported not to display enhanced mRNA levels in response to EMF exposure (Goodman *et al*, 1991).

In response to these reports, we examined similar exposure conditions with the HL-60 cell line (Krause *et al*, 1991). In addition to the dot blot methods utilized for the reported HL-60 results, we employed Northern blot protocols in order to ascertain the specificity of cDNA probes for particular mRNAs. After considerable analysis of steady state levels for *c-myc*, histone H2B and actin mRNAs, we found no consistent alteration in transcript levels that could be ascribed to electromagnetic field exposure. Thus, despite considerable effort, we could not replicate the effects reported in the literature.

Recently, it was reported by another laboratory (Czerska *et al*, 1991) that enhancements were obtained in steady state levels of *c-myc* mRNA in response to 30-180 min exposures of Daudi human lymphoma cells to 60-Hz magnetic fields of 1 G amplitude. The same exposure conditions, interestingly, failed to produce similar changes in levels of *c-myc* mRNA in the HL-60 line. In collaboration with this laboratory we have investigated the *c-myc* enhancement, and examined the response with regard to alterations in τ_{coh} parameters we previously found to influence cell response.

3.3.1 METHODS

Cell Cultures and Field Exposure. Cultures of Daudi cells were maintained in log phase growth using RPMI 1640 medium supplemented with 20% fetal bovine serum and 1% HEPES buffer. Cells were harvested and placed into fresh medium, at 7.5 x 10^5/ml, 1 hr prior to exposure. For each exposure three 75 cm^2 flasks, each containing a total of 1.13 x 10^7 cells, were exposed to the 60-Hz, 100 μT, horizontal magnetic field provided by vertical Helmholtz coils. Three identical flasks were maintained in a separate, but identical, incubator chamber as matching controls.

RNA Isolation. Isolation of RNA was accomplished by the one step-acid-guanidinium thiocyanate-phenol-chloroform extraction method of Chomczynski and Sacchi (1987) as modified by Tel-Test, Inc with the RNAzol B Method. Briefly, 7.5 x 10^6 cells in 1.5 ml micro-tubes were lysed with 1200 μl of RNAzol B by pipetting up and down 10 times and then mixing with a vortex mixer at the highest setting for 15 seconds. 120 μl chloroform (with iso-amyl alcohol in 24:1 ratio) were added and the tubes were vortexed for an additional 15 seconds at the maximum setting. The tubes were placed into an ice bath for 15 min, then centrifuged at 12,000 x g for 15 minutes at 4°C.

Six hundred microliters of the aqueous phase (upper) were carefully transferred to a clean, autoclaved 1.5 ml micro-tube without disturbing the organic phase (lower) or the interface. To this was added an equal volume of isopropyl alcohol. The tubes were briefly vortexed, placed on ice for 30 min and then centrifuged 12,000 x g for 15 minutes at 4°C. The resulting RNA pellet was washed with 1.0 ml 75% ethanol and stored at room temperature for 30 minutes. The

tubes were centrifuged for 10 minutes, 10,000 x g at 4°C. The resulting pellet was washed with 1.0 ml ice cold 70% ethanol and immediately centrifuged for 10 minutes, 7,500 x g at 4°C. The supernatant fluid was discarded and all residual liquid evaporated by spinning in a Speed Vac centrifuge for 15 minutes. The pellet was dissolved with 25 to 30 µl TE buffer(10 mM Tris-HCl, pH 7.5; 1.0 mM EDTA; 0.1% SDS) and heated at 37°C for 30 minutes. A260 and A280 values were determined and RNA samples with A260/A280 ratios below 1.8 or above 2.1 were discarded.

Hybridization with *c-myc* probe. 10 µg of RNA from each sample was prepared for gel electrophoresis by incubating it for one hour in 20 µl reaction mixtures consisting of 3.0 µl 6 M glyoxal, 8.0 µl DMSO, 2.0 µl 5X MOPS running buffer, and water at 55°C. The samples were then loaded to consecutive wells of a 1.2% agarose gel and elecrophoresed at about 50 volts for 3 to 4 hours. The RNA from the gel was transferred to a nylon membrane overnight by capillary transfer (northern blot) with 20X SSC. The RNA was fixed to the nylon membrane by drying and baking the membrane in a vacuum oven for one hour at 70 to 80°C and -25 pounds pressure.

The membrane with the RNA was prehybridized for at least 2 hours at 44°C in hybridization buffer (50% formamide; 5X SSPE; 5X Denhardt's; 0.1% SDS; 150 µg sheared and denatured salmon sperm DNA/ml) and then hybridized overnight at 44°C with ^{32}P-labeled cDNA for c-myc (Oncor). The membrane was washed in sequentially more stringent wash buffers, the last of wash being for 20 minutes in 250 ml 0.1X SSPE; 0.1% SDS at 44°C. The moist membrane was wrapped in plastic wrap and placed with Kodak XAR or XRP X-ray film overnight. Exposed bands on the X-ray film, corresponding to the proper position of the *c-myc* mRNA, were quantified by densitometry using a Bio Image Visage 60 computer system.

3.3.2. RESULTS

Densitometric data was collected from the autoradiograms of *c-myc* cDNA probed bands on the Northern blots using software which integrated the intensity over the entire band, rather than simply determining a densitometric tracing across the band. Such integrated intensities for an exposed culture and its matched control were expressed as a ratio of exposed:control, to allow comparisons to be made among samples analyzed at different times.

Time course experiments, in which cultures were exposed for 30, 60 and 180 min, consistently demonstrated increased levels of *c-myc* transcript at 90 min. Accordingly, the experiments were repeated, with multiple samples obtained at the 90 min time point. Results from the densitometric scans of the resulting autoradiograms for 8, 90 min exposures showed a small, but consistent and statistically significant increase in *c-myc* mRNA in the exposed cells. Exposed cells had a total *c-myc* mRNA level that was 1.30 ± 0.18 that of matched controls. This result reflects an alteration in *c-myc* mRNA synthesis and/or degradation that is sufficient to reflect a change in the total level of the transcript, so it would be expected that the actual rate of change in synthesis or degradation would be proportionately higher.

To determine more thoroughly the effect of the applied magnetic field on increased mRNA synthesis, cultures were exposed to the field under conditions of amplitude variation. Amplitude of the applied magnetic field was varied from 50 to 150 μT (mean field of 100 μT) at coherence time (τ_{coh}) intervals of 1.0 sec. This τ_{coh} condition has been shown by our previous work to eliminate cellular response to a field (Litovitz *et al*, 1991). With exposure conditions thus modified, 8 samples exposed for 90 min to the amplitude varied field were assayed, and found to have *c-myc* mRNA levels that were 0.82 ± 0.28 those of matched controls. Such amplitude variation thus eliminated cellular response to the field, as was observed in our work with enhancement of ornithine decarboxylase specific activity in L929 cultures.

3.3.3 DISCUSSION

Results from our exposures of the Daudi lymphoma cell line to 60-Hz magnetic fields of 100 μT are in basic agreement with the preliminary observations reported by Czerska *et al* (1991). Small, but consistent, changes in *c-myc* mRNA levels were produced as the result of exposure of the Daudi cells, but not as the result of exposure of HL-60 cells to similar fields. Further, failure of the cells to respond to fields with τ_{coh} of 1.0 sec is consistent with our previous observations of τ_{coh} effects on enhancements of ODC enzyme activities (Litovitz *et al* 1991); this result, thus, substantiates the effect of the applied magnetic field in producing changes in mRNA levels.

Resolution of the discrepancy between reports of transcriptional enhancement for several specific mRNAs, including *c-myc*, in the HL-60 cell line (Goodman *et al*, 1989, 1991; Wei *et al*, 1990), and the failure to observe such enhancment by Czerska *et al* and our group, is not clear. Although the applied magnetic field of 100 μT reported here, and also used by Czerska *et al*, is large relative to those used for other reported HL-60 results, we have employed such smaller fields with exposures of HL-60 cultures and have still obtained no alterations in mRNA levels. Hence, steady state levels of specific mRNAs appear to be altered by applied magnetic fields, but the effect is inconsistent for the HL-60 cell line from one laboratory to another. Replication of the positive HL-60 result, or demonstration of some experimental inconsistency in the techniques used to obtain it, is essential for determining more completely the enhancement of transcription during exposure to electromagnetic fields. With regard to these questions, it is important to note that Phillips *et al* (1991) recently reported both enhanced rate of transcription, using nuclear run-off assay, and steady state levels for *c-myc*, *c-fos*, *c-jun* and protein kinase C mRNAs for cultured T-lymphoblastoid cells exposed to 60-Hz, 100 μT fields. The field-induced changes in mRNAs thus appear to be relatively consistent for at least 2 cell lines, with differences among laboratories for similar results from the HL-60 cultures.

We will explore further the question of field-induced alterations in mRNAs, particularly through the use of magnetic fields in the 1 to 10 μT range, which would produce results more consistent with expected sorts of environmental exposures. Additionally, the sorts of "window" effects observed for HL60 mRNA alterations by fields (Wei *et al* 1990) may be explained by the time shifts in ODC response we noted with changes in field amplitude (Section I.E). Previous work, in which frequency and amplitude changes were examined at a single time point would not

take into account the phenomenon of a shift in the time of response, therefore producing an apparent window effect. Additional avenues of exploration will include a thorough examination of the coherence time effect with regard to *c-myc* transcription, and examination of several other mRNAs for field-induced transcriptional effects.

3.4. GENE-SPECIFIC TRANSCRIPTION STUDIES: TRANSCRIPTION RATES

Published work examining the question of electromagetic field-induced alterations in transcription of specific genes has employed dot or Northern blotting techniques which measure total, or steady state, levels of a specific mRNA. Results of such measurements have indicated a general enhancement of steady state RNA levels for *c-myc*, *fos*, β-actin and histone H2B in HL-60 cells exposed to applied 60-Hz magnetic fields for 20 min (Goodman *et al*, 1989, 1991; Wei *et al*, 1990). This sort of result is comparable to that we report here (Section II, above) for *c-myc* mRNA levels in Daudi cells. Since steady state levels of mRNA may be influenced both by rate of transcription and rate of degradation, however, the underlying basis for a field-induced increase in a specific message is unclear.

In the work reported here, relative transcription rates of *c-myc* and ß-actin RNAs were determined in both human leukemia (HL-60) and lymphoblastoid (Daudi) cell lines, and of the 45S ribosomal RNA gene in HL-60 cells. These genes were selected to assess how stringently-regulated (*c-myc*) and "housekeeping" (ß-actin, ribosomal) genes are regulated in response to ELF exposure. This work, carried out by Dr. James Greene of our group, and his associates, employed an S-1 Nuclease Protection Assay devised by him as a means to eliminate some of the problems associated with the typical nuclear run-on (run-off) assay.

3.4.1 METHODS

Transcription was determined by a novel nuclease protection assay which involved quantification of the amount of pulse-labeled nuclear RNA protected from RNase degradation by a cold hybridization probe. Briefly, cells were exposed to a horizontal, 60-Hz sinusoidally varying magnetic field of 1000 μT for 90 minutes and pulse-labeled for the final 15 minutes of exposure with ^{3}H-uridine at a concentration of 20 μCi/ml. Cells were harvested and nuclear RNA was isolated with 0.5% NP-40 lysis buffer. The nuclear RNA was then hybridized overnight with cold gene-specific oligo nucleotide probe to 45 S RNA or to plasmid based probes to *c-myc* and ß-actin. Following hybridization, the RNA was treated with S_1 nuclease, the protected RNA was precipitated by ethanol and collected on nitrocellulose filters and then quantified by scintillation counting.

3.4.2 RESULTS

The results of these determinations are shown in Table 3.7.2. For HL-60 cells, irradiation at 1000 μT for 90 minutes caused a significant (42%) enhancement in ribosomal RNA gene transcription, but not in the transcription of *c-myc* or β-actin genes. In contrast, both the *c-myc* and β-actin gene transcription were enhanced in Daudi cells. These results indicated that ELF effects on transcription are selective, capable of affecting transcription of some genes. Moreover, these results show that the transcription effects are also highly dependent on cell type.

3.4.3. DISCUSSION

These results show that rate of transcription of some, but not all, genes may be enhanced by exposure of cells to 60-Hz magnetic fields. The fact that the *c-myc* transcription rate is enhanced in Daudi cells is consistent with our work (Section 3.3, above) in which steady state levels of *c-myc* mRNA were increased by approximately 30% in Daudi cells exposed to 100 μT fields for 90 min. At least some of the increase in *c-myc* mRNA steady state levels may be attributed to an increased rate of transcription in the field. A diminished rate of degradation for the mRNA could also contribute to increases in steady state levels. Since degradation rate for the *c-myc* message has not been measured, any contribution from changes in this rate are unknown.

Although transcriptional rate changes were observed in Daudi cells exposed to the field, none were obtained for HL-60 cells assayed for the same mRNAs. The essential difference in the two cell lines that accounts for this variation is not understood. Others have reported consistent enhancement of steady state levels for both *c-myc* and β-actin mRNAs following brief exposures to 60-Hz and other ELF fields (Goodman *et al*, 1989, 1991; Wei *et al*, 1990)), whereas we have found no significant alterations in either transcription rates or steady state levels. This discrepancy is discussed in the preceeding section.

Experiments with the Daudi cell line are being repeated, using 100 μT. This field intensity will match that used for the Daudi experiments in which steady state levels of *c-myc* mRNA was measured. Preliminary results indicate that *c-myc* transcription rate is enhanced (approximately by 40%, i.e. ratio of 1.40-1.45) but that the ß-actin rate is unchanged). This sort of result is what one would predict in terms of a hypothesis by which "non-house keeping" genes, such as *c-myc* are more susceptible to perturbation by electric fields than genes such as β actin.

3.5. IDENTIFICATION OF GENES BY MOLECULAR CLONING

Another component of our research effort has been to utilize recombinant DNA approaches to identify genes whose expression is enhanced by ELF exposure. To this end, a cDNA library was constructed in λ-gt11 phage using mRNA isolated from HL-60 cells exposed to 60-Hz, 1000 μT vertical magnetic field for 90 minutes. These conditions were previously shown (Greene *et al*, 1991) to produce transient enhancement of the transcription of the 45S pre-ribosomal RNA.

3.5.1 METHODS

The mRNA used for cDNA synthesis was purified from total cellular RNA which was extracted from approximately 300 x 10^6 irradiated HL-60 cells using the guanidinium one-step procedure. Purification of the mRNA from this total cellular preparation was by two cycles of chromatography on oligo-dT sepharose spin columns obtained from Pharmacia. The poly A-RNA (mRNA fraction) was then used as template for the synthesis of the first strand cDNA with avian myeloblastosis virus reverse transcriptase. The mRNA-cDNA hybrid was then converted to the double-stranded cDNA by the method of Gubler and Hoffman (1983), using *E. coli* DNA polymerase I and RNase H as shown below in Figure 3.5. Eco RI adapters were then linked to the cDNA by T4 ligase and the cDNA cloned in the Eco RI site of λ-gt11. The recombinant DNA was packaged using the Packagene λ packaging extract (Promega). A recombinant library size of about 100,000 "clones" was constructed, representing between 80-100% of expressed genes.

3.5.2. RESULTS AND DISCUSSION

This library is currently undergoing differential screening to identify genes whose expression is enhanced by ELF exposure. The screening strategy is illustrated in Figure 3.6. It involves the screening of replica plaqued recombinant phages by hybridizing ^{32}P-labeled cDNA probes made using mRNA from exposed and cDNA probes using mRNA from control HL-60 cells. The cDNA probe synthesized using mRNA from exposed cells reflects genes expressed during the ELF exposure while cDNA probe synthesized using mRNA from control cells reflects genes expressed in the non-irradiated state. A recombinant phage that hybridizes with both probes is indicative of containing a cDNA corresponding to a consistitutively expressed gene (a gene whose expression is independent of ELF exposure) while a phage that hybridizes preferentially with the probe from ELF exposed cells would contain a cDNA that corresponds to a gene whose expression is enhanced by ELF. Only preliminary screenings have been done and these are suggestive of the library containing some differentially expressed cDNAs. During the next 6 months, differential analysis of this library is expected to be completed.

Identification and, hence, the isolation of such genes would be extremely useful in providing probes that can be used to isolate genomic versions of these genes. The genomic fragments could then be characterized molecularly, particularly with respect to upstream regulatory regions, in an attempt to discern the underlying basis for the sensitivity of these genes to ELF exposure.

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3.7 TABLES

Table 3.7.1. Coherence Time and Ornithine Decarboxylase Enhancement

τ_{coh}	ODC - AM Microwave	ODC - ELF
0.1 s	1.012 ± 0.113	0.932 ± 0.107
1.0 s	1.024 ± 0.080	0.929 ± 0.171
5.0 s	1.537 ± 0.058	1.450 ± 0.096
10.0 s	2.133 ± 0.109	1.900 ± 0.143
50.0 s	2.126 ± 0.151	2.080 ± 0.242

ODC values represent a ratio of the ODC activity of exposed cultures to matched controls. Amplitude modulation frequency of the microwave field, or the frequency of the ELF field, were switched from 55 to 65 Hz at the coherence times (τ_{coh}) indicated.

Table 3.7.2. Effect of ELF on Specific Gene Transcription

Cell Line	Irradiated transcription/control transcription		
	c-myc	β-actin	ribosomal RNA
HL-60	1.00 ± 0.08	1.08 ± 0.10	1.42 ± 0.12
Daudi	1.64 ± 0.19	1.40 ± 0.20	not done

Irradiated or control HL-60 cells were pulse-labeled for 15 minutes at 75 minutes after the start of the actual or mock exposure. The nuclear RNAs were isolated, hybridized to the appropriate cold probes, digested with S_1 nuclease, and the nuclease resistant material collected by ethanol precipitation on filters as described above. The ratios represent the counts collected on filters from the exposed sample divided by counts from the control sample after each sample counts were adjusted for the background counts. The ratios were determined from at least three experiments and errors indicated are standard errors.

3.8 FIGURES

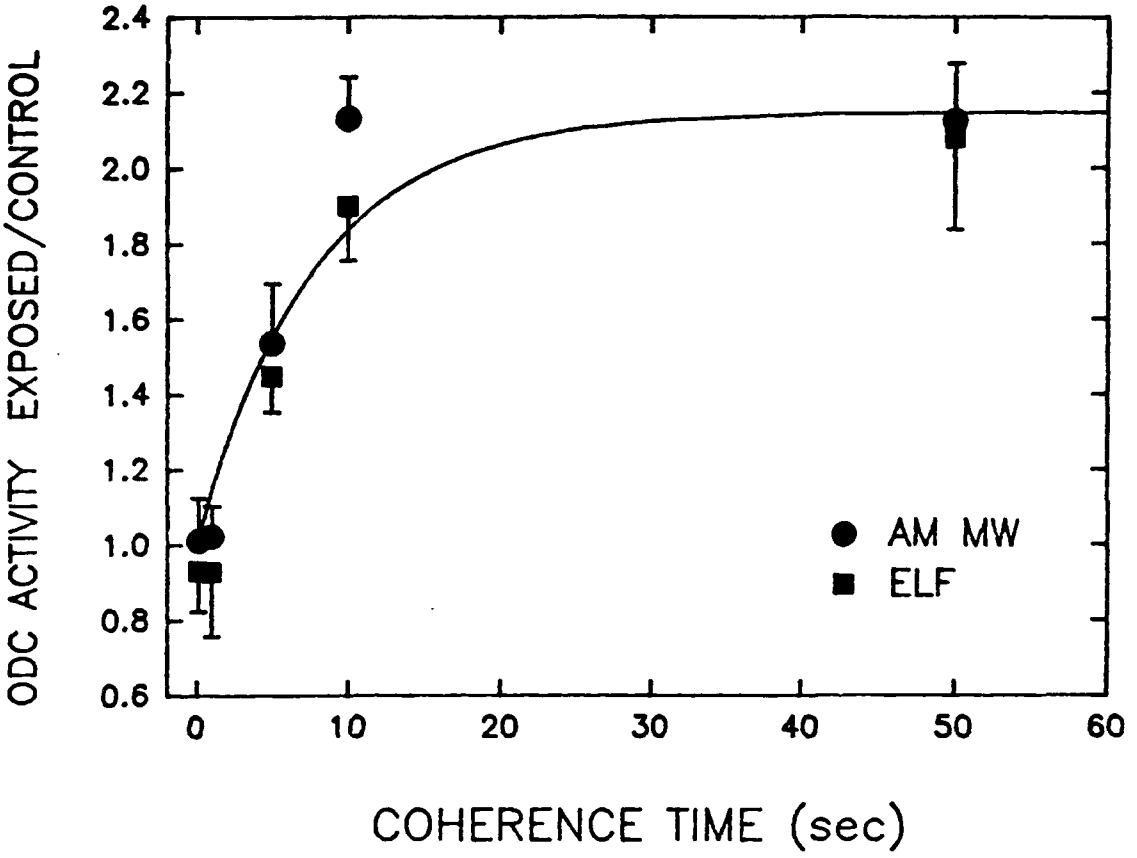

Figure 3.1. Effect of coherence time on the ODC enhancement of L929 cultures exposed either to 60-Hz amplitude modulated (23%) 915 MHz microwaves (solid circles) or a 60-Hz magnetic field of 10 μT (solid squares). The curve represents a minimum squares fit to the combined set of the two sets of experiments.

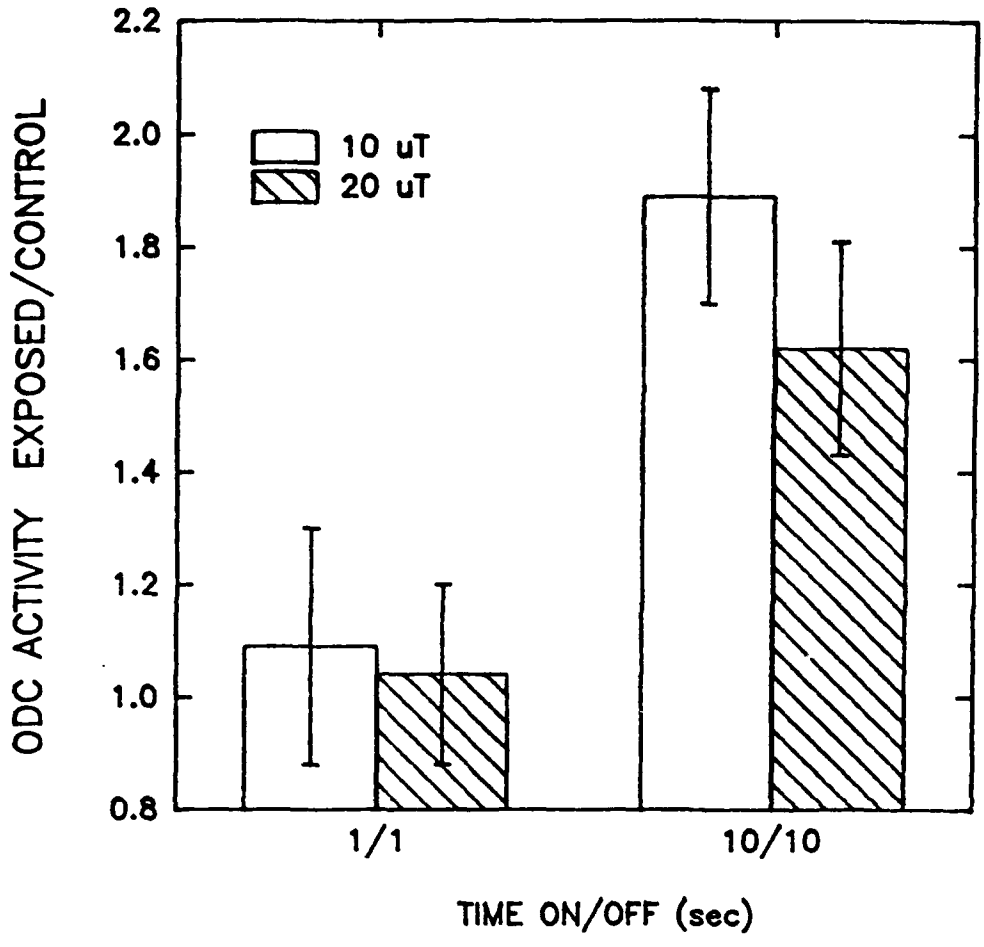

Figure 3.2. Effect of repetitive switching of a 60-Hz magnetic field on and off during the course of a 4 hr exposure of L929 cells. Switching was either 1 sec on, 1 sec off, or 10 sec on, 10 sec off. Amplitudes of 10 and 20 μT were used.

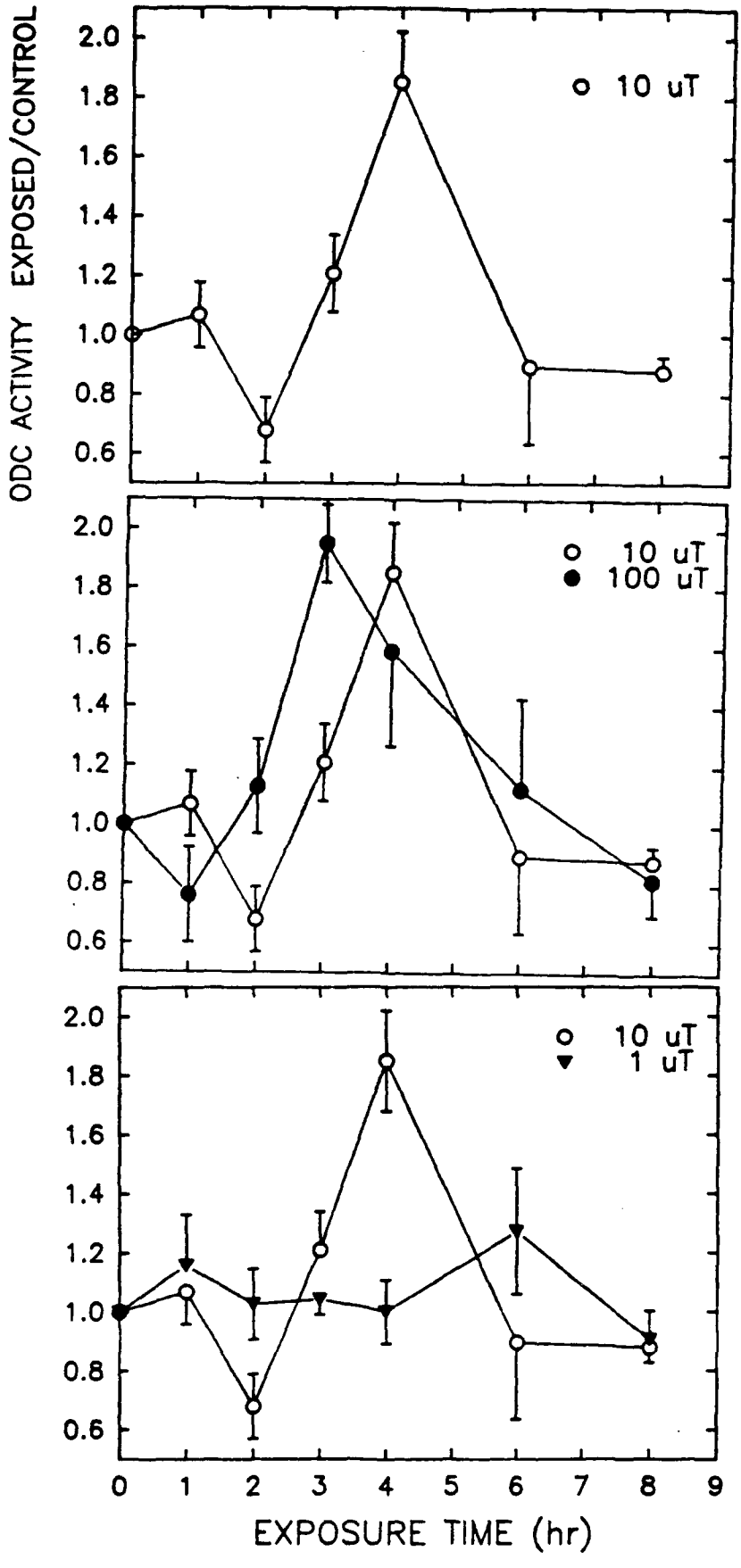

Figure 3.[3] Time course of ornithine decarboxylase enhancement relative to the amplitude of an applied 60-Hz magnetic field. (A) Field = 10 μT; (B) 10 and 100 μT; (C) 1 and 10 μT.

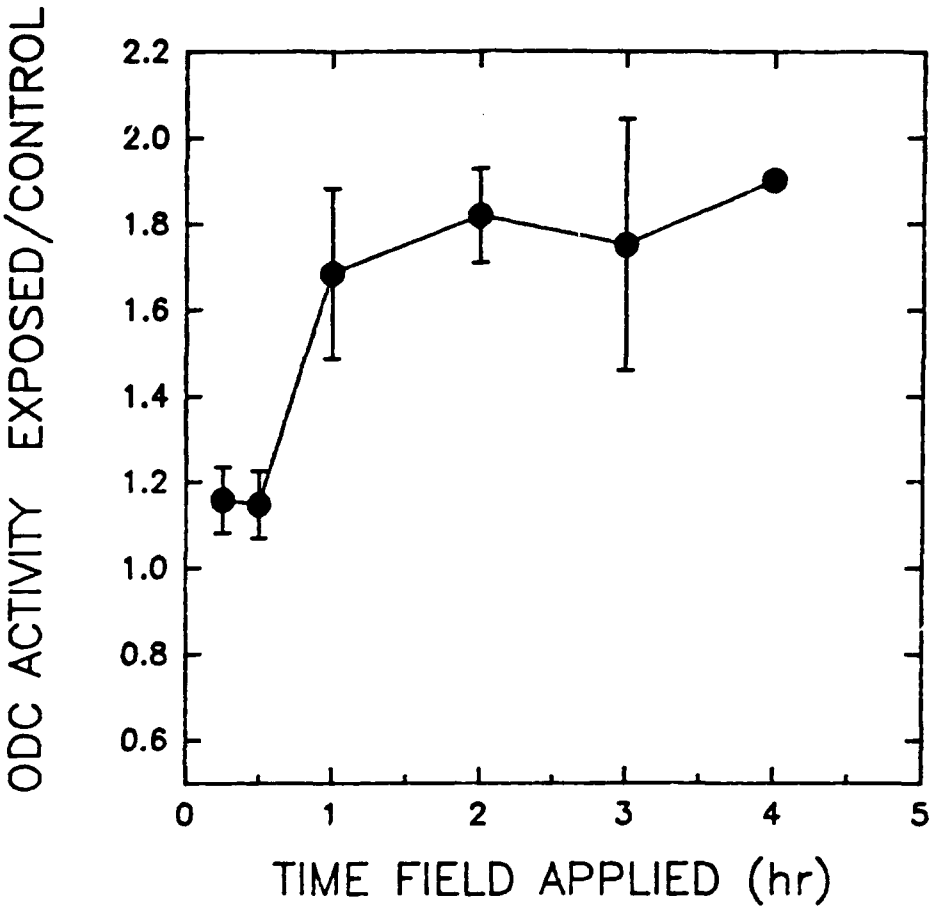

Figure 3.4. Results of a varied initial interval of exposure to a 10 μT, 60-Hz magnetic field for ODC enhancement of L929 cells harvested at 4 hr following exposure onset. ODC enhancement reached near maximal values at 4 hr following an initial exposure of 1-2 hr. Exposures of 0.25 - 0.5 produced little to no enhancement of enzyme activity 4 hr later.

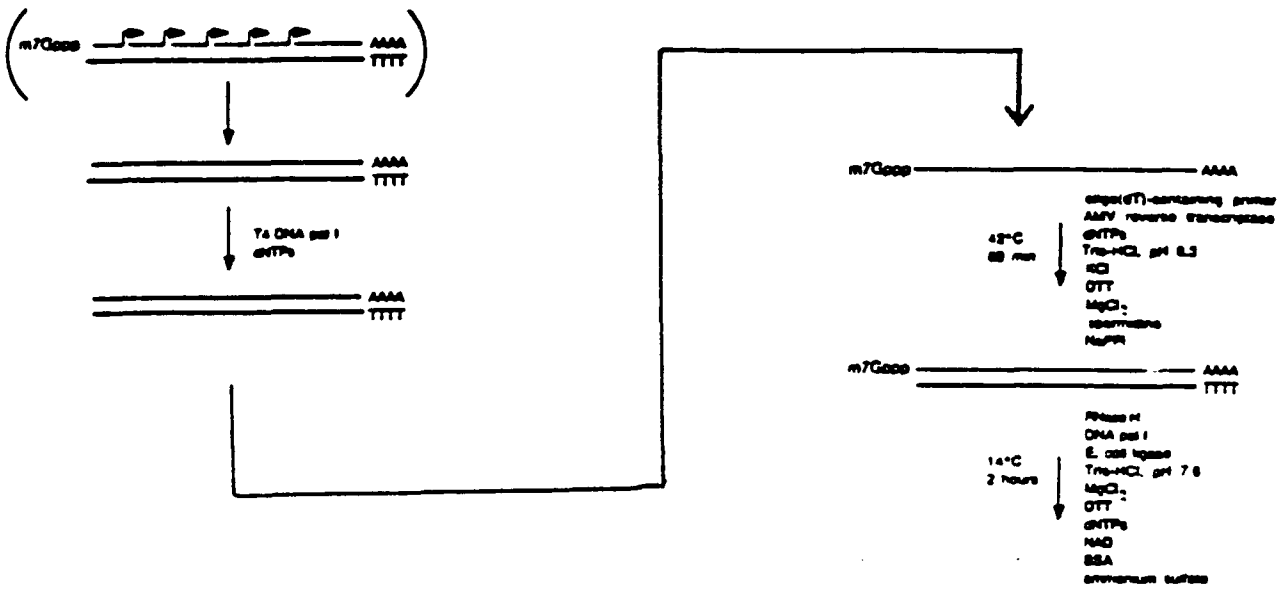

Figure 3.5. Scheme illustrating the synthesis of cDNA in experiments designed to search for electromagnetic field nhanced genes.

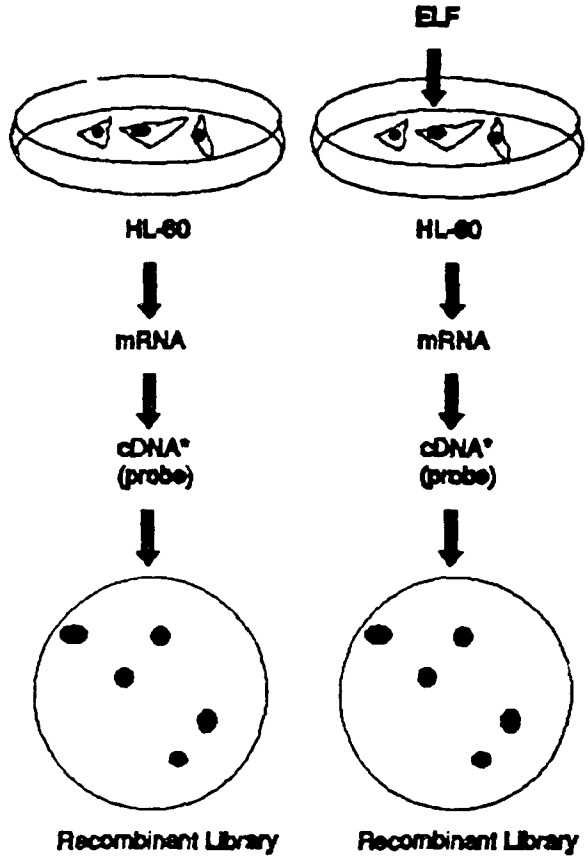

Figure 3.6. Diagram demonstrating the basic strategy to be employed in screening the recombinant libraries for genes that are selectively enhanced by exposure to 60-Hz magnetic fields.

CHAPTER 4

ENGINEERING DESIGN AND CONTROL

4.1. SUMMARY OF EFFORT

During the fourth year of this research effort the electrical engineering contribution to the program was concerned with

(1) servicing instrumentation, maintaining calibration of equipment and ensuring that procedures, where electronic equipment was concerned, were reviewed and
(2) as various additional experiments were suggested from analysis of data, new component instrumentation were developed to allow for these new measurements.

Of particular interest in this reporting period were studies which included switching of the irradiating signal in either periodic or random manner as described below.

The instrumentation actively used in the Biology laboratories during this period included:

a. One - Solenoidal ELF exposure system in which Dr. Green of the biology group studied electromagnetic field exposure effects with CW @ 60 Hz with fields mostly in the range of 100 microtesla and 1000 microtesla, some measurements were also made at 10 microtesla. The two cell systems studied were mouse HL-60 cells and human leukemia Daudi cells. Typical duration of irradiation was 90 minutes.

b. Two - Helmholtz ELF exposure systems. Dr. Mullins and Dr. Krause of the biology group studied effects of 60 Hz electromagnetic field at both CW and Switched CW. The magnetic flux density chosen for these studies were 1, 10, and 100 microtesla. The cell systems studied in these experiments were mouse fibroblast L929 and Daudi cells.

   In this case the amplitude of the magnetic flux density B was switched such that the average value of B was equal to the value used chosen for the CW case. The period of this waveform was chosen from a selection of times which included 0.1, 1, 5, 10, 20 seconds. Experiments were made with switched amplitudes ranging from approximately 100% to ±1% around the average value. The biological cells studied were L929 and Daudi. See Figure 4.1 for switching circuits.

c. One - microwave system using a Crawford cell (rectangular coaxial transmission line) in which Dr. Krause of the biology group studied the effect of approximately one gigahertz microwave electromagnetic field on L929 cells. The system was

arranged to be excited in several ways depending on the experiment under consideration:

(1) CW excitation at 0.915 GHz;

(2) Amplitude Modulation (adjusted to 23%) where, depending on the experiment, the following amplitude modulation frequencies were used; 6 Hz, 16 Hz, 55 Hz, 60 Hz, 65 Hz and 600 Hz; and

(3) a random time adjustment of the frequency associated with amplitude modulation was used to determine the effect of coherence time of the signal upon irradiation of the biological system. The percent modulation was maintained at 23%. Some measurements were also made at 60% modulation, in which case the frequency change of the amplitude modulation was from the discrete 55 Hz to 65 Hz. This small shift in frequency was shown not to be the essential factor in the resulting biological effect. The sequencing was controlled with a D/A interface which provided the desired random timing.

A recently suggested experiment to be conducted in the microwave region will be setup to simultaneously irradiate the biological cell system with both amplitude modulated microwaves superposed with switched ELF. To this end instrumentation is being developed to provide ELF fields in the 10 μT-range which accommodate to the Crawford transmission microwave cell system. The relevant schematics are shown in Figures 4.2a and 4.2b.

4.2. FIGURES

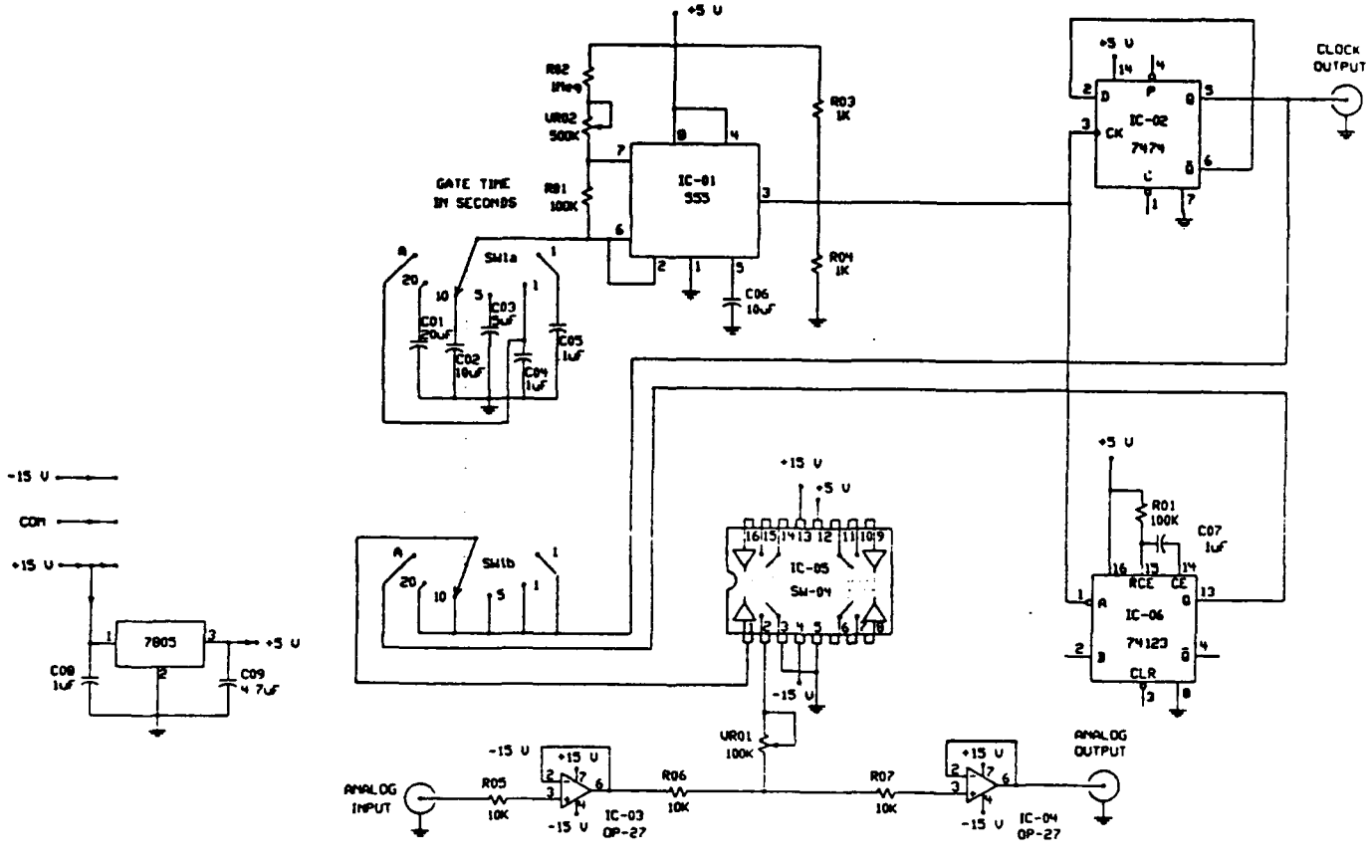

Figure 4.1. Switching Circuit for ELF Helmholtz System. Available switching times are 0.1, 1, 5, 10, 20 seconds and with 50% duty cycle. Variable amplitude in switched position from 1% to 100%.

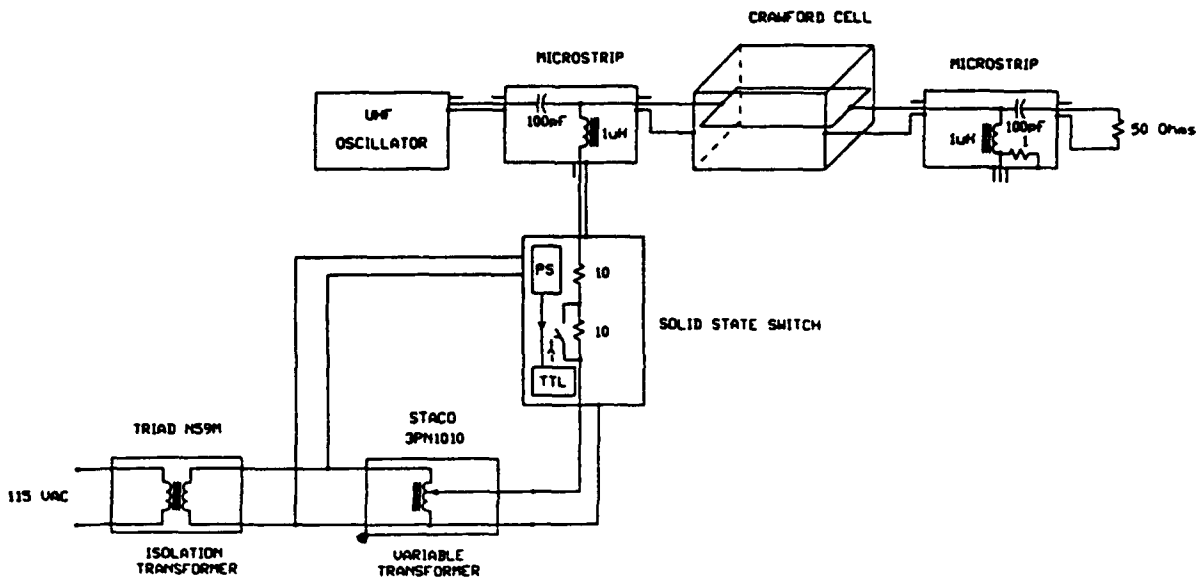

Figure 4.2a. Block diagram of ELF switching cicuit for microwave exposure Crawford cell system. The circuit provides superposition of a 60-Hz ELF magnetic field with an ELF-amplitude-modulated 0.915 GHz microwave signal.

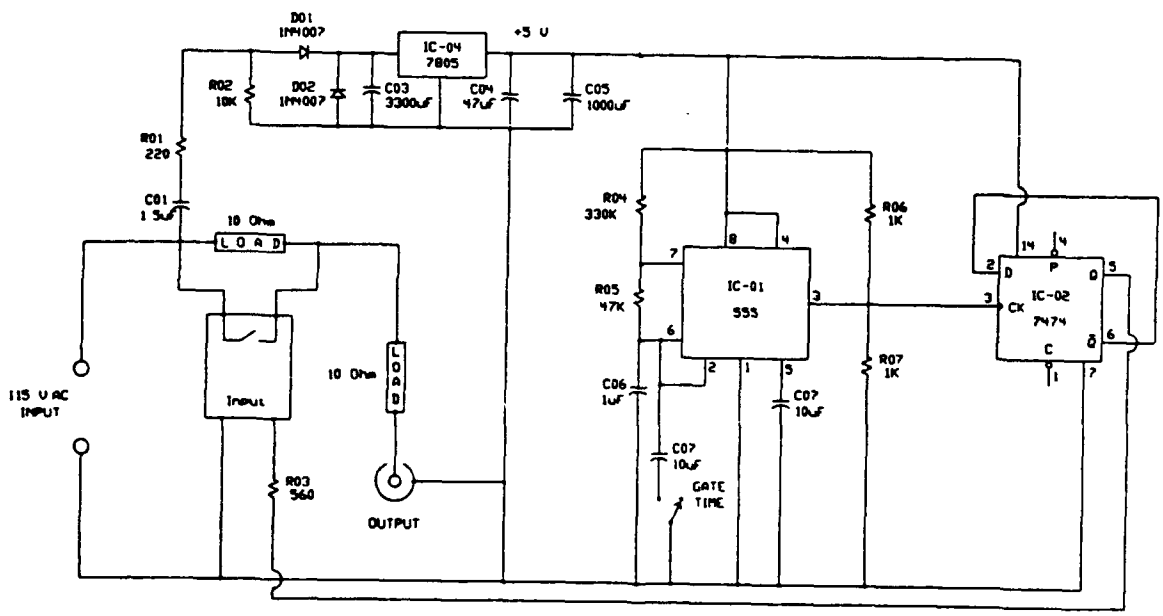

Figure 4.2b. Details of electronic switching circuiit. Two position switch allows for 1 and 10 second timing with 50% duty cycle switching.

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19. ABSTRACT (Continue on reverse if necessary and identify by block number)

The results of research directed at several of the very fundamental issues concerning cellular effects of weak electromagnetic fields is described. Despite their importance, these issues have not been satisfactorily addressed by the scientific community. They include the issues of (a) replication of experimental effects; (b) how weak electromagnetic fields can affect cells that exist in an electrically noisy environment; and (c) the explanation of complex dose-response relationships.

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19. ABSTRACT (continued from reverse side)

Our experiments unambiguously confirm the teratogenic effects of low level electromagnetic fields. The data imply that only those embryos with a genetic predisposition display a teratogenic sensitivity to electromagnetic fields. They are also definitive in establishing that exposure to ELF and AM-modulated microwave fields causes alterations in the activity of ornithine decarboxylase in various cell lines in culture.

An intriguing discovery that offers a clue to the signal-to-noise problem is the observation that EM fields applied for durations of several hours must exhibit temporal coherence for times of the order of at least 5 seconds or so if the signal transduction mechanism is to respond and bioeffects are to occur. This provides a first-level mechanism in the cell's noise discrimination process. The hypothesis that spatial coherence of the applied field is also a requirement for field-induced bioeffects provides an exclusionary mechanism that explains the cell's ability to reject endogenous thermal fields while simultaneously responding to externally impressed signals.

We have shown that electromagnetic field exposure produces a transient enhancement of both the production and degradation rates of an intermediate reaction product (mRNA) in the sequential chain of biochemical reactions occurring within the cell. The kinetic aspects of this behavior are connected to observations of "unusual" dose-response relationships such as power and exposure windows.