1982

EFFECTS OF PULSED ELECTRIC FIELDS ON MAMMALIAN CELL MEMBRANES

Guy K. Smith

EFFECTS OF PULSED ELECTRIC FIELDS ON MAMMALIAN CELL MEMBRANES

By

Guy K. Smith

B.S., Cornell University, 1974

Thesis

submitted in partial fulfillment of the requirements

for the Degree of Doctor of Philosophy

in the Department of Biophysics

at the Medical College of Virginia

Health Sciences Division

Virginia Commonwealth University

Richmond, Virginia

April, 1982

This thesis by Guy K. Smith is accepted in its present form as satisfying the thesis requirements for the degree of Doctor of Philosophy.

Date: 29 April 1982

Approved: [redacted]

or

Chairman of Graduate Committee

29 April 1982 [redacted]

[redacted]

[redacted]

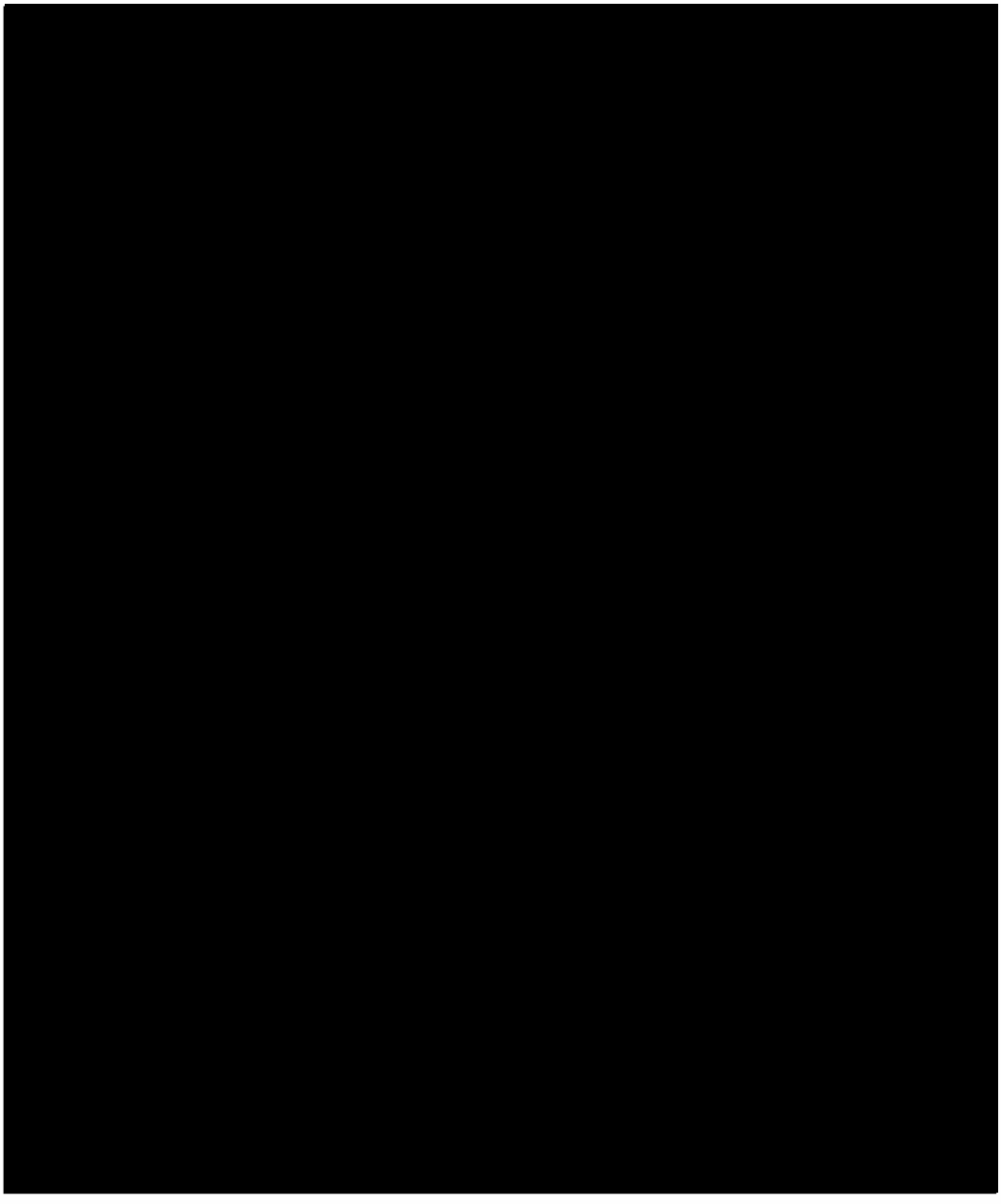
29 April, 1982. [redacted]

29 April, 1982 [redacted]

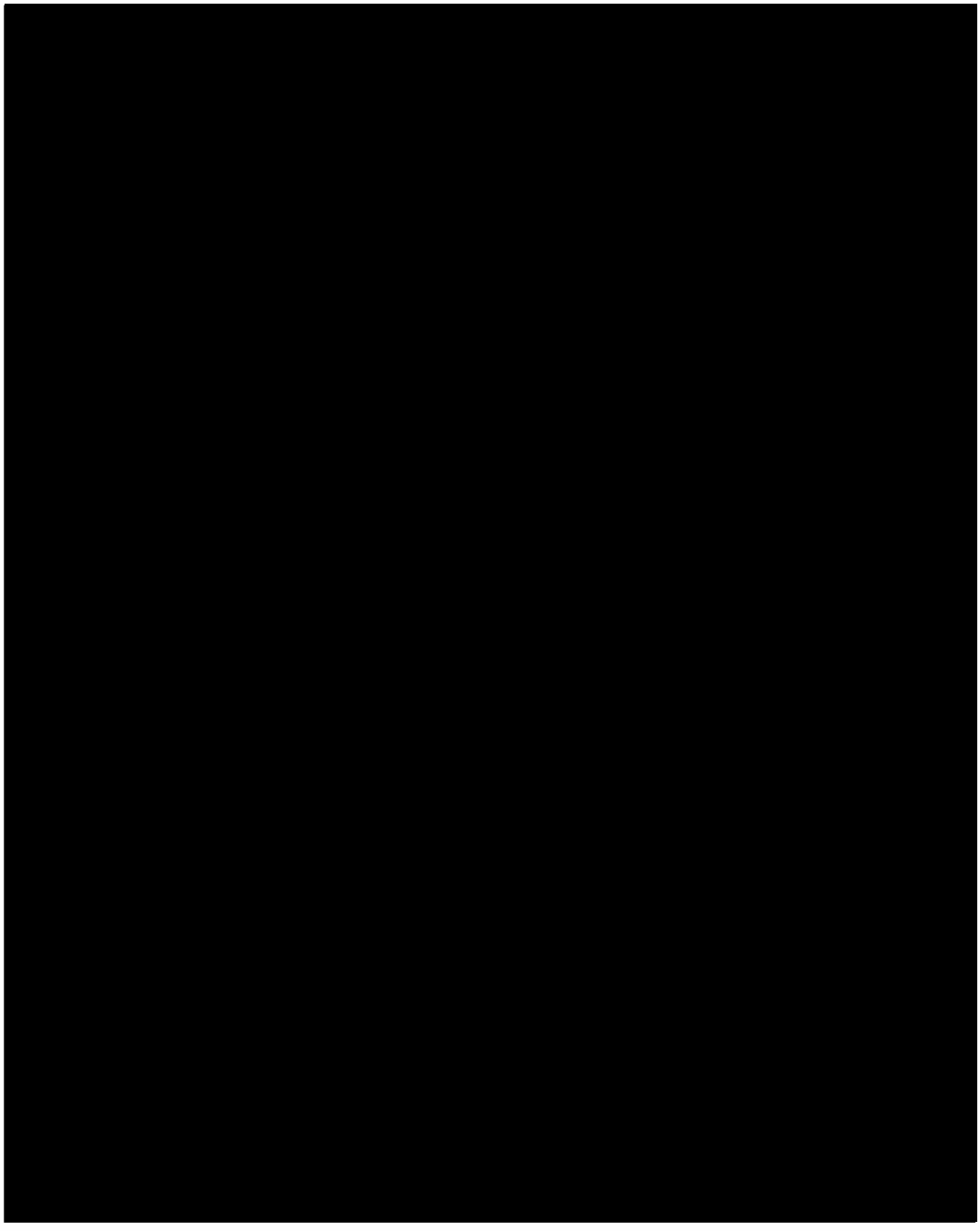
APPROVED: [redacted]

Chairman, MCV Graduate Council
Dean, School of Basic Sciences

CURRICULUM VITAE



BIBLIOGRAPHY



DEDICATION

To my Dad,

who could not be here to share the joy of this achievement.

ACKNOWLEDGMENTS

First and foremost, I would like to acknowledge and publicly thank my wife, Genevieve, for her patience, understanding and support throughout this belated pursuit of a dream. Her love and her faith in me made it possible to get through the hard times and finally achieve this long-held goal. There is no doubt in my mind that she really does deserve "half of the degree."

Special thanks to Dr. Allen M. Kaplan and his "crew," especially Kevin, Sandy, and Ann who graciously shared their lab with me. Without Dr. Kaplan's moral and financial support this project might never have been completed. I would also like to thank Dr. Daniel Watts, Dean of Basic Sciences, for having sufficient faith in my ability to provide the necessary financial assistance when the situation looked bleak, and for an occasional, well-timed kick, where it was needed.

Thanks too, to my parents Winona K. Smith and the late Edward C. Smith who instilled in me the self confidence and perseverence needed to complete this undertaking.

I should also like to thank Dr. Li Ming Liu for his patience while teaching me electromagnetics, and for many helpful discussions on the interaction between electromagnetic fields and biological systems. Thanks also, to

my advisor, Dr. Stephen F. Cleary, the other members of my committee, and Dr. William T. Ham, for the many lessons taught formally and informally as well as those taught indirectly by example.

A special thanks to Mrs. Fay Akers for doing an exceptional job typing this manuscript.

Financial support was provided by: the Office of Naval Research, the NIH, Biological Resources Program, an A.D. Williams Fellowship, and the Department of Biophysics.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

EFFECTS OF PULSED ELECTRIC FIELDS ON MAMMALIAN CELL MEMBRANES.

Guy K. Smith, Ph.D.

Medical College of Virginia/
Virginia Commonwealth University, 1982

Major Professor: Stephen F. Cleary, Ph.D.

The effect of pulsed electric fields on cell membranes were studied to investigate the effects of an electric field, per se, on biological systems, without the densometric and other technical problems associated with other forms of nonionizing radiation. Exposure of mouse splenocytes to a high voltage pulse resulted in an increase in membrane permeability to K^+ that was dependent on both the electric field strength and the pulse duration. Exposure to a 2 μsec, 3.0 kV/cm pulse elicited a 50% loss of intracellular K^+ indicating that the critical transmembrane potential (V_m) at breakdown was 1.26 volts for the membrane of mouse spleen cells. These results agreed with previous studies on erythrocytes and micro-organisms.

Effects of a pulsed electric field on a cell's functional integrity were assessed by measuring ^3H-thymidine incorporation by lymphocytes cultured in the

presence and absence of various mitogens following exposure to an electrical pulse. No statistically significant effects on the response of mouse spleen lymphocytes to Con-A, PHA, or LPS were observed following exposure to a 2 μsec electric pulse. Exposure to 10 μsec pulses $\geq$ 2.4 kV/cm produced a statistically significant reduction in the response of lymphocytes to LPS stimulation. While not statistically significant, results from both 2 and 10 μsec experiments consistently indicated that exposure to pulses at sublethal electric field strengths may have a stimulatory effect on mouse spleen lymphocytes. This result is discussed and an experiment to resolve this issue is presented.

Exposure of Ehrlich ascites tumor cells to 2 μsec electrical pulses produced no statistically significant alterations in the tumorigenicity of these cells. K^+ release data indicated these cells are unusually resistant to the effects of pulsed electric fields having a high breakdown potential, V_m = 2.37 volts.

INTRODUCTION

Since the early studies of electrical phenomena, the interaction of electricity and biological systems has been a subject of great interest, yet there is still limited understanding of the basic mechanisms through which an electric field exerts its influence on living organisms. This study was undertaken to investigate the earliest and most basic consequences of the interaction between an electric field and cells, and to delineate some of the subcellular mechanisms involved in this interaction. The motivation for undertaking this study at this time was due, in part, to the technical advances facilitating the study, and to recent reports of investigations into the effects of pulsed electric fields on microbial systems (1-4) and mammalian erythrocytes (3-6).

An electric field consisting of a single DC pulse, microseconds in duration has been used for this study. Such fields eliminated effects due to heating of the sample, as well as other ambiguities associated with field modulation, polarization and other factors encountered with microwave and radiofrequency electromagnetic energy. In order to circumvent the problem of interpretation associated with the unique structural aspects of the erythrocyte membrane, and to extend the

relevance of this and previous studies with pulsed electric fields to eukaryotic cells in general, it was decided to study mammalian leukocytes. Lymphocytes obtained from the mouse spleen were selected as the primary cell for this investigation since they are relatively easy to obtain in the requisite numbers 10^8-10^9 cells/experiment). In addition, the splenic lymphocyte is an extensively studied, "typical" eukaryotic cell with known biological functions which facilitate the assay of its physiological integrity.

Current interest in electromagnetic effects on biological systems comes from several related but separate points of view. One concern relates to the potential hazards associated with the increasing "electromagnetic pollution" of our environment. This concern is currently focused on the biological effects of microwave and radiofrequency radiation (7-9), and with possible health hazards associated with high voltage transmission lines (10,259). The fact that the biological effects associated with electric fields are subtle in nature has led to a highly polarized controversy regarding both the validity of some of the data and the pathological significance of the reported effects. However, recent results seem to indicate that they are, in fact, biologically significant effects associated with exposure to electric fields (11-13).

Another aspect of electrical hazards is concerned with accidents involving lightning and other high voltage sources. These encounters are catastrophic, resulting in death or very severe burns. The burns associated with such incidents form a distinct class of severe burns, different and frequently more difficult to treat than conventional heat-induced burns (14,15). Great success in treating severe electrical burns has been achieved in recent years with the recognition of some of the differences between electrical and heat-induced burns, and the accompanying alterations of burn treatment regimes to compensate for these differences. Still, a number of the problems encountered in treating severe electrical burns persist, particularly the long-term, latent effects involving tissue degeneration long after apparent recovery (14,15).

Apart from the concern for the hazards associated with exposure to electromagnetic fields, there is long standing interest in the perturbation and control of biological organisms by electromagnetic fields of internal or external origin. The most obvious example being the nervous system of higher animals. Indeed, the current level of understanding regarding the interaction of electrical pulses with biological systems is due largely to the extensive electrophysiological investigations into the generation and conduction of nerve

impulses. However, it has become more apparent that electrical fields are involved in a more general sense in the regulatory and control functions of living systems (16-21). Although this should not be surprising considering the ionic nature of the biological milieu, the significance of bioelectrical phenomena have only recently begun to be recognized. The results from studies of electrically mediated growth phenomena including electrically stimulated limb regeneration in amphibians (16) and related studies on the differentiation of "terminal" post-mitotic cells (22) have resulted in a resurgence of interest in bioelectromagnetic phenomena. One practical aspect of this renewed interest in electricity mediated growth is the successful clinical application of electrical fields to induce healing of bone fractures that had failed to respond to conventional treatments (23,24). Another application is the recent clinical utilization of microwave induced hyperthermia as an adjunct to cancer chemo- and radiation therapy (25,26).

The studies on electrically mediated cell growth and differentiation are particularly relevant to the present study in which lymphocytes exposed to electric field strengths below the membrane breakdown threshold may have exhibited some degree of growth stimulation or enhanced mitogenic response. One possible mechanism for

electric field induced stimulation of lymphocyte proliferation is suggested by recent studies on the changes in transmembrane cation fluxes in cells prior to the initiation of DNA synthesis and mitosis (27-34). Thus, an investigation of the effects of pulsed electric fields on lymphocytes is relevant to some of the most basic questions in cell biology in addition to providing a better understanding of interactions between biological systems and electromagnetic fields, per se.

Effects of Pulsed Electric Fields on Cells

The effects of pulsed electric fields on the cell membrane of erythrocytes, micro-organisms, and model lipid bilayer membranes (BLM) have been the subject of a number of investigations beginning in 1967 and 1968 with the studies of Sale and Hamilton into the lethal effects of pulsed high voltage electric fields on micro-organisms (1-3). These studies were exceptional in terms of breadth and completeness, and while seldom given more than passing acknowledgement, most of the subsequent studies have merely corroborated and extended Sale and Hamilton's work.

The object of Sale and Hamilton's studies was to determine if a nonthermal bactericidal effect resulted from exposure to pulsed electrical fields, and to

explore the mechanisms producing such an effect. The experiments were carried out using a small rectangular exposure chamber with parallel plate carbon electrodes mounted in a water cooled brass block. The electrodes were separated by polyethylene spacers of varying thickness. Sale and Hamilton used a 10 kV square wave pulse generator to expose their samples to electric field strengths of up to 25 kV/cm using 1-99 pulses with pulse widths from 2-10 μsec at a repitition rate of 1 pulse per second. Both the current and the voltage across the sample were monitored by means of a dual-beam oscilloscope. The effects of DC pulses on nine species of bacteria and two strains of yeast at various stages of growth were investigated in their initial studies. These studies were eventually extended to include bovine and equine erythrocytes. Lethal effects were demonstrated on all types of micro-organisms studied, with each type exhibiting different sensitivity to the pulsed electric field (RBC's > yeasts > vegetative bacteria >> spores). It was shown that the lethal effects were not due to products of electrolysis, nor to heating of the sample. The lack of a thermal effect on survival was demonstrated by exposing bacteria to pulsed field in media of different conductivities and plotting the percent survival as a function of current density,

calories per cm^3, field strength, and total exposure time. Plots of percent survival as a function of current density (A/cm^2) and calories per cm^3 both gave a disperse scatter of points; whereas, plots of percent survival as a function of field strength (kV/cm) and total exposure time (pulse width x number of pulses) resulted in single curves with all experimental points falling close to the curves (1). This clearly demonstrated that the electric field strength was the significant parameter rather than Joulian's heating of the sample.

Sale and Hamilton further demonstrated that electric field induced lethality was due to the loss of the semipermeable barrier properties of the cells' plasma membrane. This loss of membrane integrity was demonstrated by the lysis of red blood cells and protoplasts, and by the leakage of intracellular contents from lethally exposed bacteria, as indicated by the presence of ninhydren positive material in the suspending medium and by an increase in the medium's light absorption at a wave length of 260 nm, characteristic of nucleic acids. Membrane damage was also indicated by loss of the ability of E. coli to plasmolyze in hypertonic medium, and by the release of β-galactosidase activity by a permease-negative E. coli mutant. The DC electrical pulses also

caused loss of motility and loss of the ability of vegetative bacteria to synthesize the inducible enzyme β-galactosidase. The fact that these effects were not due to the direct action of the electric field on the protein molecules was indicated by the lack of inhibition of cellular or membrane bound enzymes in isolated enzyme preparations, even after exposure to field strengths up to 30 kV/cm. Electron microscopic examination of bacteria exposed to lethal field strengths showed no detectable alteration of the bilayer structure of the cell membrane (2).

Using a simple spherical model of a cell Sale and Hamilton calculated the electric field induced transmembrane potential taking the membrane breakdown point as the electric field strength producing 50% survival for a particular cell type. The transmembrane potential at breakdown was approximately 1 volt (range 0.7-1.15 volts) for all the cells studied. A plot of the cumulative size distribution relating the relative number of cells larger than a given diameter had the same shape as a plot of the percent lysis as a function of field strength (kV/cm), in agreement with the model used to calculate the field induced transmembrane voltage. Sale and Hamilton suggested that the loss of the barrier

properties of the cell membrane was due to electrical field induced conformational changes or phase transition in the membrane (2,3).

Neumann and Rosenheck (35) and Rosenheck, et al. (36) investigated the effects of pulsed electric fields on chromaffin granules as an in vitro model system to study possible neuroendocrine secretory mechanisms which might be operative under the influence of nerve impulses. In these studies a suspension of chromaffin granules was exposed to electrical impulses of up to 25 kV/cm with an exponential time constant of approximately 150 μsec. Permeability changes produced by the electric field were determined by ultraviolet (UV) absorption spectrometry of catecholamine and ATP released into the suspending medium. The permeability changes induced by the electric field were short lived (on the order of msecs) and completely reversible. Because of the small size of these vesicles the electric field induced transmembrane potential was calculated to fall into a range of from 25 mV to a maximum of 457 mV (37,38). On the basis of this relatively low transmembrane potential and the transient nature of the catecholamine-ATP release it was concluded that dielectric breakdown (a phenomenon described below) was an unlikely mechanism for the permeability changes observed. As an alternative, a polarization mechanism

involving the field induced redistribution of the vesicular counter-ion cloud was suggested. In the later paper, Rosenheck et al. (36) studied light scattering and fluorescence changes during and after exposure of suspensions of chromaffin granules and membranes from osmotically lysed granules to pulsed DC electrical fields. These studies indicated that changes in the optical properties of chromaffin granules reached a maximum in approximately 150 μsec, and was followed by a relaxation having at least two decay components (τ_1 ≃ 1 msec, τ_2 ≃ 30 msec). Calculations indicated that all of the observed signals originated from the granule membrane, and the observed changes could be accounted for by relatively small changes in the membrane refractive index or thickness, or by changes in the particle size. This study also indicated that catecholamine release occurs during the first few milliseconds of the transient field induced changes in membrane permeability (36).

A completely new, nonoptical method for counting and sizing particles and cells was introduced by Coulter in 1953 (39). This technique was automatic, accurate, statistically more reliable, and less tedious than traditional microscopic examination. The basic principle of a Coulter type transducer is to monitor changes

in the resistance across a small diameter orifice as dielectric particles pass through the orifice. Generally, this is accomplished by imposing a constant current across a small diameter orifice in a conducting fluid, then as a particle, with electrical resistance different from that of the suspending electrolyte, passes through the orifice it displaces electrolyte producing a change in the resistance between the electrodes. By Ohm's law this resistance pulse is observed as a voltage pulse that is amplified and measured with a multichannel pulse height analyzer or counted with an electronic scaler. According to Coulter, the observed voltage pulses were directly proportional to the particle volume (39,40). However, later studies indicated that this is not strictly true as indicated by skewed blood cell distributions and other anomalies. A number of theoretical and experimental studies revealed the limitations of the standard Coulter counter arrangement (39,41-44), and have led to the development of several different means of correcting these problems (45-48). The major problem of Coulter's system is the unrestricted path followed by particles as they pass through the orifice. Particles passing through the orifice via some off axis path will experience different hydrodynamic and electrical fields than those moving through the central axial "core" of

the orifice, resulting in the production of pulses of different shape, width, and amplitude by physically identical particles. This problem can be reduced by using a longer orifice (43), or eliminated by restricting the particles to the central axis of the orifice by using hydrodynamic focusing (45-47). In general, hydrodynamic focusing eliminates artifactual nonsymmetric distributions; however, it was observed that the use of high electrical field strengths in a hydrodynamically focused Coulter counter resulted in a volume distribution profile that was skewed toward lower volumes when compared with the profile obtained from the same population of cells sized at lower field strength. Zimmerman attributed this underestimation of cell size to the dielectric breakdown of the plasma membrane as the cells passed through the electric field (4,49).

In order to understand this process of dielectric breakdown of cell membranes Zimmermann launched an extensive investigation into the effects of pulsed external electric fields on cell membranes (4). Zimmermann and his colleagues have studied pulsed electric field effects on suspensions of E. coli (49), human and bovine erythrocytes (4,50-60), various plant cells and protoplasts (56-59) directly on single cells using Valonia utricularis (61-63), and on lipid bilayer membranes (64-66).

Valonia utricularis is a large marine algal cell (2-3 mm in diameter x 3-5 mm in length) which does not have a cell wall. Consequently, Coster and Zimmermann were able to insert stimulating and measuring electrodes into the cell and measure, directly, the transmembrane potential induced by 500 μsec current pulses. These studies showed that the potential needed to produce electrical breakdown of the membrane of V. utricularis was .85 volts at 20°C. It was further determined that this was a true threshold as multiple subcritical pulses did not cause breakdown. This critical potential (V_c) was strongly temperature dependent, decreasing from 1000 mV at 4°C to 64 mV at 30°C. Dielectric breakdown in these cells occurred in 1-5 μsec, was not the result of local heating, and was completely reversible if a period of 10 seconds was allowed between pulses (61-63). If the intrapulse duration was less than 10 seconds each subsequent pulse caused a further decrease in membrane resistance to a final value of about 30 Ω, after which no further electrical breakdown could be detected. The relatively short time course of these events and the strong temperature dependence lead Coster and Zimmermann to conclude that the nonlinearity of the current-voltage (I-V) curves observed in these studies was due to

electrical breakdown of the membrane and not "Punch-Through,"[1] an alternative effect observed by Coster in earlier studies with Chara australis and Nitella sp. (67). The I-V curves and breakdown could be accounted for by considering the compression of the membrane due to stress set up by the electric field in the membrane (63). This was done by adapting Crowley's electro-mechanical model for the electrical rupture of lipid bilayer membranes (68) to cell membranes.

Zimmermann, et al. have also made an extensive series of studies of dielectric breakdown in human and bovine erythrocytes (4,38,50-58) using a hydrodynamically focused Coulter counter. In this series of experiments the Laplace equation was used to theoretically predict the breakdown voltage. In parallel "impulse" experiments it was determined that the combination electrical breakdown and subsequent osmotic processes were responsible for the release of intracellular material. This contention is supported by the finding that in both human and bovine red blood cells the redistribution of sodium and potassium occurred at the breakdown field strength (52). In human RBC's the

---

1. "Punch-through," electrostriction and other models accounting for the dielectric breakdown of cell membranes are discussed in greater detail below.

critical field strength for breakdown occurred between 2 and 3 kV/cm, corresponding to a transmembrane potential of 1 volt, regardless of the composition of the suspending medium. In contrast, the electrical hemolysis (i.e. field-induced hemoglobin release was very sensitive to the extracellular environment. The onset of hemolysis for human erythrocytes in isotonic NaCl or KCl solutions was observed at 4 kV/cm with 50% hemolysis at 6 kV/cm, while the inclusion of phosphate or sulfate anions, sucrose, EDTA, or inulin in the cell suspension medium increased both of these values to higher field strengths. This was especially true for EDTA and inulin which caused a shift in the onset of hemolysis to 14 kV/cm. In contrast, bovine erythrocytes experienced dielectric breakdown of the membrane at 4-5 kV/cm, and dielectric breakdown was directly coupled to hemoglobin release. The electrical hemolysis of bovine erythrocytes was also unaffected by changes in the composition of the suspending medium with the exception of inulin which prevented hemolysis even at 15 kV/cm, the highest field strength used (51). The reason for this species difference is unclear, and these authors' only suggestion was that it may be related to differences in the membrane composition. This contention is supported by another series of experiments using benzyl alcohol to

alter the fluidity of erythrocyte membranes (53). In this study, Zimmermann and colleagues were able to demonstrate the sensitivity of dielectric breakdown to changes in membrane fluidity with an increase in membrane fluidity resulting in an increase in V_c, the critical membrane voltage (53).

These results from studies on erythrocytes combined with the earlier studies with bacteria, and those obtained with V. utricularis have lead Zimmermann to conclude that electromechanical dielectric breakdown is a generalized cell membrane phenomenon (4). Zimmermann has further extended this model of electro-mechanical coupling across a cell membrane to the field of plant physiology where it has been suggested as a mechanism for the sensing and osmoregulation of turgor pressure in plants (4,56,57).

On the basis of their work on the Coulter counter, Zimmerman et al. have developed a new multiparameter cell characterizing system that measures the volume, internal conductivity and breakdown potential of cells (58,59,69). In this system a voltage ramp is applied across the orifice as it is traversed by a cell. At the electrical breakdown voltage there is a decrease in the orifice resistance that is detected as a decrease in the ramp voltage. The size distribution is obtained from

the detector response at initial subcritical field strengths while the inflection point in the voltage curve provides the breakdown voltage, and the resistance at that point can be analyzed to give the intracellular conductivity (58). The previous studies on red cell membrane fluidity (53) and differences observed in the dielectric breakdown threshold of lymphocytes and lymphoblasts (70) indicate that this may prove to be a useful tool in the study of membrane physiology, the characterization of cell populations (60,69), and monitoring cellular changes produced by experimental perturbations (71).

Zimmermann and coworkers have also investigated a number of other applications of dielectric breakdown of cell membranes. These application studies included an extensive investigation into the preparation and characteristics of red blood cell ghosts (50,54), including hemoglobin free ghosts (55). They have used the techniques developed in these studies as a basis for producing drug loaded erythrocytes and lymphocytes as a potential drug delivery systems for treating tumors and other diseases (71-75). These studies are particularly significant as they include a systematic study of some of the electric breakdown properties of lymphocytes (75). Zimmermann et al., observed a critical field

strength of 2.5 kV/cm above which a hydrodynamically focused Coulter counter underestimates the mean size of mouse thymocytes. These cells were also reported to show potassium and sodium exchange with the external medium when exposed to a single 2.0 kV/cm, 40 μsec pulse; although no significant morphological damage was observed at the electron microscopic level when compared with micrographs of control cells. Electron micrographs of thymocytes exposed to a 40 μsec 3 kV/cm pulse then incubated for 30 minutes at 37°C indicated that substantial irreversible structural changes had occurred in the cells. Exposure to 4 and 5 kV/cm, 40 μsec pulsed fields produced increasing levels of structural damage. Vacuolation of the cytoplasm and damage to the nuclear membrane and neucloplasm appear in all of the cells exposed to field strengths from 3 to 5 kV/cm (pulse length 40 μsec) (75). In view of these results Zimmermann et al. explored the effects of shorter pulses in order to find exposure conditions that would produce reversible increases in the thymocyte membrane permeability without effecting the intracellular structure. These studies indicated that pulses of 0.5 μsec would produce the reversible changes in the cell membrane needed to permit entrapment of solutes added to the external solution prior to the application of the pulsed

electric field. This was demonstrated with eosin uptake in mouse thymocytes exposed to 0.5 μsec electrical impulse at 4°C at various field strengths followed by incubation at 4°C for 10 minutes then at 37°C for 30 minutes. Under these conditions eosin uptake was observed from 8-20 kV/cm with 50% uptake at 12 kV/cm. These authors also demonstrate that the thymocytes resealed in less than 5 minutes at 37°C, as indicated by eosin uptake (75).

Beginning from a different point of view, Tsong and his colleagues have conducted a parallel series of studies into the effects of pulsed electric fields on human erythrocytes (5,76-84). These investigations were begun to study the relaxation properties of human erythrocyte membranes and hemolysis using the temperature jump technique of Eigen and deMaeyer (89). Initially, the observed swelling and hemolysis was attributed to the rapid change in temperature due to Joule heating of the cell suspension. It was thought that such heating might produce a temperature difference across the membrane leading to changes in the general colligative properties of the solution such that water would be forced into the cell due to the osmotic pressure effect, $\Delta\pi = cR\Delta T$ where c = salt concentration, R = the gas constant, and ΔT = transmembrane temperature difference.

Another possible mechanism considered by Tsong et al. at this time was the "thermal osmosis effect" discussed by Katchalsky and Curran (85). However, before this phase of their study was finished, their data (5,77) and the reports from other labs (3,4) clearly indicated the dependence of membrane breakdown on the electrical field strength. During the course of these early T-jump experiments Tsong et al. (77) used turbidity measurements to monitor changes in the human red blood cell suspensions, and were able to obtain relaxation time constants for the alterations in cell volume and membrane integrity as follows:

τ_1 = 20 μsec,

τ_2 = 5 msec,

τ_{lag} = 0.1 sec,

τ_3 = 0.55 sec.

These time constants were attributed to the relaxation of membrane structures (τ_1 and τ_2), the membrane rupture (τ_{lag}), and the hemolysis reaction (τ_3). Erythrocyte ghosts produced three different relaxation times while erythrocytes from clinical patients with leukemia, dystrophy, and sickle cell anemia had fast relaxation times (τ_1, τ_2) similar to normal cells, but the slower relaxations (τ_{lag}, τ_3) were sensitive to the physical alterations in the cells associated with the different disease states (77).

These studies were followed by an elegant series of experiments by Kinosita and Tsong (5,78-82) who demonstrated that the exposure of cells to a pulsed high voltage electric field induced the formation of "pores" in the cell membrane. By varying the ionic strength of the "pulsation" medium and, consequently, the temperature jumps experienced by the cell suspension they demonstrated that the electrical field strength and not the increase in the temperature of the cell suspension caused the change in membrane permeability (5). This was demonstrated graphically by plotting the percent hemolysis as a function of the temperature change and as a function of the electrical field strength. The former plot resulted in a separate curve for each ionic strength tested; in contrast, the graph of percent hemolysis versus electrical field strength resulted in all of the data forming a single curve indicating that the electric field strength was the major parameter responsible for the observed changes in membrane permeability. Kinosita and Tsong's data indicated that 50% hemolysis required a temperature jump of 2°C in isotonic saline while a temperature increase of 0.07°C produced the same effect in 3% isotonic NaCl:97% iso-osmotic sucrose. In both cases the field strength was 2.5 kV/cm, which corresponds to a transmembrane potential of approximately 1 volt (5). These authors proceeded to demonstrate that

hemolysis was always preceded by an increase in membrane permeability to potassium and sodium that continued over a period of several hours. This implied that the observed hemolysis was a secondary effect due to the colloid osmotic swelling of the cells that followed the dramatic increase in membrane permeability to water, potassium, sodium and other small molecules which occurred in less than a microsecond after the potential across the membrane reached the critical level of about one volt (79).

Kinosita and Tsong took the view that this initial increase in permeability was caused by the induction of pores in the plasma membrane by the external electric field. This idea was supported by the observation that large molecules (e.g. BSA, stachyose, and to a lesser extent, sucrose) could prevent this swelling (78). Using a series of carbohydrates of known molecular size, they were able to show that the prevention of cell swelling and the uptake of radioactively-labeled carbohydrate was a function of the average molecular radius of the carbohydrate used. Furthermore, it was shown that the pore size formed in the cell membrane could be increased by increasing either the field strength or pulse duration or by decreasing the ionic strength of the pulsing medium (78,79). It was observed that the

field-induced pores would reseal if the cells were incubated at 37°C for 20 to 60 minutes and that the resealed "ghosts" were able to accumulate potassium and extrude sodium against a concentration gradient. Kinosita and Tsong suggested that the ability to produce pores of controlled size in erythrocyte membranes made red cells treated with pulsed electromagnetic fields potentially attractive as an immunologically compatible alternative to liposomes as drug carriers (78,80). To test this possibility RBC's from AKR/j mice were exposed to a single 3.5 kV, 80 μsec pulse then incubated with [^{14}C]sucrose for 1 and 6 hours then these [^{14}C]-sucrose loaded erythrocyte "ghosts" were injected back into homologous mice via the tail vein. These mice were then monitored for ^{14}C in their blood, and it was found that more than 85% of the loaded RBCs survived with a half life close to that of normal mouse red cells (14 days compared to 15-18 days) (80).

Kinosita and Tsong (82) then investigated the dynamics of electrical field-induced pore formation by studying the time dependent changes in the conductivity of cell suspensions during and following a high voltage pulse. Defining the conductivity, κ, of the sample by:

$$\kappa = (I/V)\ (1/A) \qquad I$$

where I is the total current, V is the applied voltage, 1 is the sample thickness (i.e., distance between electrodes), and A is the cross sectional area of the sample. By monitoring changes in I and V during the initial pulse and during a second pulse, and by measuring κ directly at low voltages after 1 msec they were able to follow the changes in membrane conductivity. After correcting for the increase in κ due to heating of the sample these authors observed a threshold of 1.5 kV/cm for a time dependent increase in conductivity of the suspension. This threshold was independent of the ionic strength of the medium and showed little change with temperature of the sample or with the rise time of the applied field. At field strengths $\geq$ 3 kV/cm the following series of changes were observed:

a) a 1 μsec increase in the membrane conductance to 10 S/cm^2;

b) a 100 μs increase in membrane conductance to 100 S/cm^2;

c) after the pulse terminated the membrane conductance dropped to a level close to its original value ($<10^{-1}$ S/cm^2);

d) after about 10 sec. the conductivity of the suspension again started to increase.

Kinosita and Tsong attributed the latter change to colloid osmotic swelling of the cells caused by the osmotic imbalance created during the initial, fast, voltage dependent formation of pores in the cell membrane (82). In recent papers (83), Teissie and Tsong suggest that about 35% of the field-induced pores involved Na/K-ATPase while the larger fraction of pores are formed at other unidentified sites on the cell membrane.

Kaibara and Tsong (84) have reported that 20 μsec electrical pulses of 0.8 kV/cm applied to a suspension of sickle erythrocytes changed the membrane permeability so as to facilitate the permeation of oxygen and the unsickling of treated cells at a faster rate than unpulsed cells following the reintroduction of oxygen. This effect on the sickle cell membranes persisted for about 3 hours at 25°C. No Na^+ or K^+ leakage nor any osmotic swelling was detected in the sickle erythrocytes treated with the 20 μsec 0.8 kV/cm pulses; although osmotic swelling was observed at field strengths of 1.0 kV/cm. It was also observed that sickle cell hemolysis was produced at lower field strengths than in normal erythrocytes (1.5 kV/cm compared to 2.2 kV/cm for 50% hemolysis). This lowered breakdown voltage was insensitive to oxygen tension in contrast to the dependence

of the breakdown voltage on cell shape, cell volume, and media composition previously observed for normal red cells (5,51,79,84).

Studies on the biological effects of pulsed electric fields were initiated by Cleary and Hoffman (6,86) by exposing erythrocytes to multiple exponential pulses. These studies indicate possible species differences in the field strength or pulse length needed to induce potassium efflux or hemolysis, with the sensitivity of dog red cells being less than rabbit red cells that were less sensitive than human erythrocytes (6). Potassium efflux was observed to be the most sensitive indicator of membrane alterations followed by osmotic fragility and hemolysis, respectively. A pulse duration threshold for producing membrane damage was observed to be between 0.43 μsec and 0.75 μsec. Pulse duration dependent field strength thresholds were also observed. These were true thresholds in that even prolonged exposures to thousands of pulses at subcritical field strengths or subcritical pulse lengths produce no detectable differences in external K^+ or hemoglobin concentrations, compared to control suspensions. A comparison of K^+ loss, osmotic fragility, and hemolysis as a function of the number of pulses a sample was exposed to indicated that once the field strength and pulse duration thresholds for potassium

release were exceeded each successive pulse produced more damage, progressively weakening the cell membrane (6). Cleary and Hoffman interpreted this data as indicating a multi-step mechanism producing membrane damage. This mechanisms consists of an initial opening of pores in the membrane by the electric field followed by an enlarging of these pores by subsequent pulses, possibly accompanied by the opening of new pores in the membrane (6,86). These observations and the proposed multi-step mechanism are consistent with the observations and suggested mechanisms from other laboratories, as previously cited.

Effects of Pulsed Electric Fields on Model Membranes

Related studies on the electrically induced breakdown of membranes employing artificial lipid bilayer membranes (BLM) as model systems have been undertaken in a number of laboratories (6,64-66,88). Cleary and Hoffman were able to demonstrate that for oxidized cholesterol bilayers the amplitude of a voltage pulse needed to rupture a BLM varied inversely with the log of the pulse duration. This log-linear relationship was shown to hold at least over the pulse duration range from 10 msec (355 mV) to 30 μsec (640 mV) (6). This result is in good agreement with more recent studies by Benz and Zimmermann (64,65).

Recently, a number of laboratories have adapted electrochemical relaxation techniques (89-91) to the study of charge transport and other electrical properties of bilayer membranes (92). Sargent used a voltage jump technique (93) to study the time course of small voltage induced structural changes in bilayer membranes. Sargent was able to determine the time constants (τ) and amplitudes of dielectric relaxations ($\Delta C/C_o$) in oxidized cholesterol bilayer membranes at 21°C:

a) τ_1 = 3.3 msec, $\frac{\Delta C}{C_o}$ = 0.8% and τ_2 = 0.7 msec, $\frac{\Delta C}{C_o}$ = 0.6%, reorientation of dipole clusters in the plane of the membrane;

b) τ_2 = 155 μsec, $\frac{\Delta C}{C_o}$ = 1.5-3%, rotational reorientation of individual molecules;

c) τ_3 = 18 μsec, $\frac{\Delta C}{C_o}$ = 1.4%, small amplitude reorientations of individual dipoles about an axis in the membrane.

Here C_o is the membrane capacity at zero voltage, and the "relaxation amplitude" ($\frac{\Delta C}{C_o}$) is the percent change in capacitance which "is related to the 'dielectric increment' through the dielectric constant" (88). The time constants observed for electrostrictive effects were between 2 and 50 msec as determined from 250 mV voltage pulses which caused membrane breakdown after a few seconds. This complex time course was not observed with

pulse amplitudes of 125 or 200 mV which produced a response that was well characterized by a single exponential function (88).

Benz and Zimmermann (64-66) have employed the charge-pulse technique to study the electrical breakdown of BLMs. This technique developed by Benz and Läuger (94) and others (92) to study ionic transport in bilayer membranes takes advantage of the RC nature of such membranes. The membrane's capacitance (Cm) is charged with a short current pulse (of the order of μsec or less) and the resulting voltage across the membrane is monitored by an external measuring circuit as it decays through the membrane resistance (Rm) (64,92). Using this technique to study oxidized cholesterol BLMs Benz and Zimmermann made a number of interesting observations. First, in agreement with previous studies (6,68,95), they found that relatively long (msec to sec) voltage pulses between 150 and 400 mV cause the membrane to undergo irreversible, mechanical breakdown. This mechanical breakdown is similar to that occurring at the end of the lifetime of an artificial bilayer membrane, and seems to be related to the composition of the membrane (96-98). It is interesting to note that this breakdown process did not occur instantaneously, rather it developed slowly, with an initial period of about 250 μsec after the end of the charging pulse during which

there was a gradual decay of voltage. This initial phase ended with a shorter period during which the voltage dropped sharply to zero. This period varied between 50 and 150 μsec depending on the amplitude of the initial pulse, with higher initial voltages having shorter discharge times (64).

In contrast to these findings, Benz and Zimmermann observed that much higher voltages could be applied to the membranes using current pulses of 100-400 nsec duration to charge the membrane. In these cases an electrical breakdown occurred at about 1,000 mV as indicated by the rapid discharge of the membrane potential to a value below the mechanical breakdown threshold. This electrical breakdown was completely reversible and reproducible with as many as 20 successive pulses being applied to the same membrane. This electrical breakdown is characterized by a sudden change in the conductance state of the membrane with the resistance across the membrane dropping from 5×10^9 Ω to 5 Ω in less than 10 nsec after reaching the critical breakdown voltage (V_c). This high conductance state prevents the membrane from being charged to voltages higher than V_c so that even current densities as high as 2.6 A/cm^2 could be used without causing irreversible mechanical breakdown (64). The V_c was determined for both solutions containing a

variety of mono- and divalent ions at various concentrations, and it was observed that the breakdown voltages were about the same (V_c = 100 mV) for all the combinations tried with the exception of 0.1 mKCl (V_c > 1500 mV) and $MgSO_4$ (V_c = 1700 mV), the only 2:2 valence combination used (64). The breakdown voltage of oxidized cholesterol bilayer membranes was also observed to be temperature dependent: ≃ 1500 mV at 4°C, ≃ 1200 mV at 25°C, ≃ 800 mV at 40°C, and ≃ 500 mV at 48°C. These values were valid for charging times from 10-800 nsec. For charging pulses of 0.8--10 μsec the breakdown voltage varied as an inverse function of the pulse duration reaching a plateau at about 10 μsec (V_c 20° = 500 mV, V_c 40° = 400 mV). This plateau voltage is approximately 50 mV above the voltage producing mechanical breakdown with pulse lengths on the order of 100 μsec (64,65). BLMs formed from a variety of other lipids (dioleoyl phosphatidylcholine, egg phosphatedylethanolamine, monoolein, phosphatidylcholine/cholesterol mixture, egg lecithin, and brain phosphatidylserine) were observed to exhibit relaxation behavior very similar to that of the oxidized cholesterol membranes; however, only membranes formed from oxidized cholesterol or 2:1 oxidized cholesterol/lipid exhibited reversible breakdown. All other membranes ruputured after exposure to supracritical voltage pulses (64-66).

By using two simultaneous pulses, a short high voltage charging pulse and a longer low amplitude voltage monitor pulse, Benz and Zimmermann were able to follow the resealing process in oxidized cholesterol and 2:1 oxidized cholesterol/lipid membranes. At temperatures from 2°C to 20°C the decrease in membrane conductance during resealing could be fitted with a single exponential curve with time constants of 10 and 2 μsec, respectively. This data indicated an activation energy for resealing of about 50 kJ/mol, which could indicate a diffusion controlled process. This data can be explained in terms of a high electric field-induced pore formation model. Calculations based on this assumption indicate the formation of 10^7 field-induced pores per cm^2 with an initial pore radius of 4 nm, implying a diffusion constant on the order of 10^{-8} cm^2/sec for lateral diffusion of the lipid (66). The resealing data above 25°C result in more complex experimental curves requiring at least two exponential decays to fit the data. For example, experiments at 50°C yield rate constants of 350 μsec and 2 μsec (72).

Benz and Zimmermann (64,65) have interpreted the results of these "charge pulse" experiments on lipid bilayer membranes as suggesting another alternative or

co-mechanism of electrical breakdown. These authors point out that the intense electric fields associated with dielectric breakdown of membranes may impart sufficient Born energy,[2] approximately 10^5 J/mole, to the ions in solution to inject those ions across the lipid-aqueous solution interface of the membrane (99). This is even more likely if the membrane is thinned by the electrostrictive forces associated with the electric field (64,65).

A team of Russian scientists recently published the results of an extensive theoretical and experimental study of electric breakdown in bilayer lipid membranes (104,112-118). The experimental part of this study involved the development of two different dynamic techniques and their applications to the study of BLM model membrane systems (104,112). As a result of these experiments Chizmadzhev, Abidor, and their colleagues have suggested that electrical breakdown of membranes is

---

2. The "Born charging energy" or the "self energy" is the leading term in the equation giving the energy of a charged particle in a given medium, formally:

$$\Delta F = \frac{q^2}{2\varepsilon a} \qquad \text{II}$$

where q is the charge, a is the radius of the particle, and ε is the dielectric constant of the medium. The difference of this term in aquions solution and in the membrane must be provided to move an ion across the membrane-solution interface (99,100).

due to the local deformation of the membrane leading to the formation of defects in the form of pores (tens to hundreds of angstroms in diameter). These defects are veiwed as nucleation centers analogous to those associated with phase transition phenomena.

These investigators have made a number of interesting observations including the finding that, if the perturbing electric field is turned off when the first conductance changes are seen the membrane will settle into a metastable state lasting minutes to hours, then either relaxing into its original state or rupturing (104). It has also been observed that the addition of UO_2^{+2} ions to the bath solutions results in the apparent spontaneous formation of through-going pores in the membrane even in the absence of an electric field. This both increases the current through the membrane which becomes electrically invisible and stabilizes the membrane; although, if a high voltage is maintained across the membrane for a long enough time the membrane will collapse (104). Another interesting aspect of this study is the development of the concept that the formation of local defects in the membrane is caused by the membrane's internal electric field (104,112). The kinetic aspects of these experimental results have been

explored theoretically (115,116), and a stochastic model of the process developed (117,118). Finally and perhaps the most exciting result of this study is the suggestion that the local defects formed in the membrane are "hydrophilic inverted pores," making this a truly molecular level model (104,112).

Mechanisms of Dielectric Breakdown in Lipid Membranes

Several different mechanisms have been purposed to explain the breakdown of cell membranes under the influence of high electric fields. At this point in time there is a general consensus that the observed effects of pulsed electric fields are the result of a complex multi-step process that can, for the sake of convenience, be divided into primary, secondary, and tertiary effects (6,75,79,82,86):

1) primary, field-induced effects - attributed to the direct influence of the electric field on the structure and function of the membrane;
2) secondary effects that result from the locally high electric field and high current density in local regions of the membrane following the initial breakdown of the membrane in those regions (e.g. local heating, electrophoresis effects, etc.);

3) tertiary effects that effect both the cell membrane and the cytoplasm; principally osmotic processes resulting from the increased membrane permeability.

However, in terms of a more detailed mechanistic picture of the primary process of a pulsed electric field interacting with a cell membrane there is no such consensus. A number of possibilities have been suggested and discarded, and a few other theories are currently under consideration.

Of the theories that have been discarded because they did not fit the experimental data, the most complete and interesting is the suggestion that "Punch-through" was involved in the electrical breakdown of cell membranes (61,67). Coster (67) developed this theory as an explanation for the reversible electrical breakdown he observed when studying the electrical properties of the algal cells, C. australis and Nitella sp. While plotting the I-V characteristics of the membranes of C. australis, using a current scanning technique, Coster observed that for a sufficiently large negative (i.e. hyperpolarizing) potentials the current suddenly increased very rapidly. Although, this was very similar to dielectric breakdown it was not destructive as on removal of the potential the membrane

returned to its normal state and the whole I-V scan could be made repeatedly giving the same curve including the breakdown at the same voltage every time. Coster observed that this was very much like the electrical breakdown phenomenon that occur in reverse bias solid state P-N junction diodes. This is known as "the punch-through" effect. Modeling the cell membrane as a double fixed charge lattice separated by a depletion layer, Coster was able to account for the electrical characteristics of C. australis and Nitella sp. including the observed breakdown which by analogy to solid state physics can be referred to as "Punch-through" (67). This phenomenon is associated with ionic currents and changes in ion profiles in the membrane, processes that have time constants on the order of 0.01 to 1 sec (62). As the breakdown phenomena being considered here and that observed by Coster and Zimmermann (61,62,69) in V. utricularis occur in a microsecond or less, "Punch-through" is not a plausible mechanism.

On the basis of the experiments Sale and Hamilton (1) and Kinosita and Tsong (5), and the small temperature increases observed by all the investigators in the field, thermal effects can be eliminated as possible causes of electrical breakdown. This view is further supported by the relatively linear I-V curves obtained

from V. utricularis (61) as opposed to the extremly nonlinear relationship that would be expected if localized thermal membrane damage was the primary mechanism involved in the dielectric breakdown of cell membranes.

One possible mechanism producing membrane instability in the presence of a high electric field is Crowley's electromechanical model (68). Crowley pointed out that in the presence of a strong electric field a bilayer lipid membrane would experience a compression due to the electrostrictive force associated with the applied field. Under the influence of this field dependent compression the membrane thickness would decrease until at some point it would reach a critical thickness and collapse (68). This model has received considerable criticism, and is generally viewed as an inadequate description of the behavior of a BLM in an electric field (95,101-104). The problem with Crowley's model stems in part from the simplifying assumptions that were made, particularly the failure to consider the viscoelastic forces in the membrane and the assumption that the transverse compressive elastic modulus would be constant over the whole range of compression. The later assumption proved to be particularly bad in light of recent evidence that the commonly observed voltage dependent change in the specific capacity (i.e. thickness) of bilayers is due almost entirely to the "squeezing

out" of solvent in the commonly used Mueller-Rudin type membranes (97). In "solvent free" Montal-Langmuir type lipid bilayers the voltage dependent change in specific capacitance is extremely small (-0.02%) (105,106).

Ironically, Zimmermann and his colleagues have adapted Crowley's model into the best experimentally supported model of dielectric breakdown in cell membranes (4,51,57,61-63,87,107-111). These authors have even extended the concepts of electro-mechanical coupling inherent in this model to the problems of sensing and regulation of osmotic and turgor pressure by plants (55,56,107). Zimmermann et al. were aware of the short comings of Crowley's model as applied to BLMs, but reasoned that cell membranes were much more heterogeneous than BLMs and adopted the idea of electromechanical stress to explain their observations on electrically induced breakdown of cell membranes. In its simplest form this model (63,109) is based on the assumption that a definite membrane area can be regarded as an isotropic elastic dielectric between the two sides of a parallel plate capacitor formed by the membrane solution interfaces. At equilibrium the electrical compressive force, P_e, arising from the intrinsic membrane potential or from an externally applied electric

field is counter balanced by the elastic restoring force P_m, generated by the compression of the membrane:

$$P_e + P_m = 0 \qquad \text{III}$$

A quantitative description of the electrical compressive and elastic restoring force is difficult at the molecular level, so it is assumed that the macroscopic laws of electrostatics and elasticity (Hooke's Law for the one-dimensional case) are applicable. Thus the electric compressive force is given by:

$$P_e = -\frac{d}{ds} \int_0^{\delta} \frac{1}{2} \varepsilon \varepsilon_o E^2 \, dx \qquad \text{IV}$$

and the elastic restoring force is:

$$P_m = Y_m \int_{\delta_o}^{\delta} \frac{dx}{x} = Ym \ln\left(\frac{\delta}{\delta_o}\right) \qquad \text{V}$$

where ε is the dielectric constant or relative electric permitivity, ε_o is the electric permitivity of free space, E is the electric field strength, δ is the membrane thickness of the stressed state, δ_o the thickness of the unstressed membrane (i.e. V = 0). Y_m is the elastic compressive modulus transverse to the plane of the membrane (Young's modulus). This is a very complex term and as a first approximation was assumed to be independent of δ in the derivation of equation V.

If the field within the membrane is independent of position, x, then Eq IV reduces to:

$$P_e = + \frac{\varepsilon \varepsilon_o V^2}{2\delta^2} \qquad \text{VI}$$

where V is the transmembrane potential.

Substituting Eqs V and VI into Eq III yields Eq VII.

$$\frac{\varepsilon \varepsilon_o V^2}{2\delta^2} = -Y_m \ln\left(\frac{\delta}{\delta_o}\right) \qquad \text{VII}$$

As V becomes large and δ becomes small a point is reached where the electric compressive force is increasing more rapidly with decreasing δ than the elastic restoring force, i.e.:

$$\frac{\partial P_e}{\partial \delta} > \frac{\partial P_m}{\partial \delta} \qquad \text{VIII}$$

At this point catastrophic collapse of the membrane will occur (63). Coster and Zimmerman indicate the reduction in δ at this point is about 39%. They also indicate that for large compressions a cell membrane is unlikely to have linear elastic properties (63). Nevertheless, this electromechanical model is generally in good agreement with the experimental results, particularly recent refinements of the model which deal with the probable nonlinearity of Y_m and other visoelastic considerations in a more realistic manner (108).

Despite the relative success of Zimmermann's modification of Crowley's electromechanical model it still leaves much to be desired (102). Being a macroscopic

theory it gives little insight into the molecular aspects of the problem, and has little predictive value. The problem of a local mechanism has been addressed by a number of investigators. Hamilton and Sale suggested the possibility of a phase transition involving the rotation of the membrane phospholipids (1-3), as did Rosenheck et al. (36). Kinosita and Tsong (5,78-82), and Cleary and Hoffman (6,86), suggested the formation of pores in the membrane, and Tessie and Tsong indicated the possible involvement of the membrane bound Na/K ATPase in the electrical breakdown of cell membranes (83). Recently, Benz and Zimmermann pointed out that ions in a strong electric field, such as that associated with membrane breakdown, would gain sufficient "Born energy" to be injected across the aqueous solution-membrane interfacial energy barrier, and this might account for the various observations of the electrical breakdown of cell membranes, especially if the membrane was also experiencing electric compression (64,65). Still another alternative is the statistical mechanical model developed by Chizmadzhev, Abidor, et al. This model, based on an extensive experimental investigation, suggests that hydrophilic inverted pores are formed in the membrane by the formation of local defects which act as nucleation centers analogous to those involved in phase transition

phenomena. In this model the local defects are caused by the electrical field induced in the membrane's interior by the applied field (104,112).

Thus, at this point in time we have a fairly successful macroscopic model of the electrically induced breakdown of cell membrane integrity (109), and a generally agreed upon understanding of the tertiary consequences of this loss of membrane integrity (i.e. colloid osmotic lysis) (5,79). However, a more detailed understanding of the primary and secondary processes involved in breakdown at the molecular level has yet to emerge. There are a number of suggestions warranting further consideration includng the energetic consequences of the applied electric field (64), the possibility of electric field-induced phase transitions (119), and the formation of local defects leading to pore formation and subsequently, to mechanical breakdown of the membrane (104). None of these possibilities has been rigorously examined or experimentally tested in terms of electric breakdown of cell membranes.

None of the above models provide a clear mechanistic picture of the interaction between a cell and an imposed, transient electric field, nor do any of these models have overwhelming evidence to support its case.

It also seems unlikely that an accurate understanding of the molecular events involved in this interaction will be coming forth in the near future. This is even more evident when the experimental difficulties, and the sophistication of the experimental technique involved in monitoring molecular events in such a system are considered. Being cognizant of this situation, the investigation presented here was not designed to validate any of the proposed models nor to develop a "new" mechanism; rather, its purpose was to provide additional information on the cell physiological aspects of the cell-pulsed electric field interaction. Within this context the present investigation also addressed the question of the applicability of the observation from previous studies to mammalian cells, in general. This question is particularly important as all of the previous studies on pulsed electric fields have, with one exception (75), been directed to erythrocytes, micro-organisms, and plant cells. The cell selected as a "typical" mammalian cell was the mouse spleen lymphocyte. In this context, it is important to examine the current state of knowledge concerning the structure and function of lymphocytes and to explore the available information on the effect of other physical modalities on lymphocytes.

Lymphocytes

Lymphocytes in the blood were described, and their origin in the lymphocytic system was recognized in a series of papers by William Hewson in the 1770's (120, 121). This early microscopist made an extensive comparative study of lymphatic systems which culminated in a treatise published after Hewson's premature death. In this treatise Hewson described the anatomic structure of the lymphatic organs, (lymph nodes, spleen, and thymus), and attempted to relate the anatomic structure of these organs to their physiological function. This description includes Hewson's astute and amazing, for the time, speculation that the thymus was a distribution organ in hemopoiesis (121). The identification of the lymphocyte as a distinct cell type was first made by James in 1846 (121). This designation of the lymphocyte as an independent cell type was firmly established in 1879 by Ehrlich, using his differential staining techniques (120,121). Shortly thereafter, an enormous rift developed between those who believed the lymphocyte to be a multipotential stem cell giving rise to other cell types and the other major school of thought which held

this to be a futureless end-cell or at best a trephocytic cell which broke down to provide other cells with nutrient during tissue repair (121). Neither of these points of view was ever established, and the ubiquitous little cell's functional significance remained a mystery until about 1960 (120-122). In the late 1950's and early 1960's the direct descendency of the antibody secreting plasma cell from the small lymphocyte was established (120-122), and the role of lymphocytes in cell mediated immune reactions began to emerge (120,121). "In other words," immunology discovered that "lymphocytes are the immune system" (122).

With this impetus, the next two decades saw an astounding proliferation of studies on lymphocytes. The result is a body of literature far too vast to be adequately considered in this short review. Paradoxically, the difficulties in obtaining pure lymphocyte preparations and the preoccupation with the immunological aspects of lymphocytes have created a situation where some aspects of lymphocytes have been studied in great detail while there is still relatively little knowledge of the basic cell biology of lymphocytes (123,124). Consequently, this section will be confined to a very brief review of some basic aspects of lymphocyte structure

and function that are particularly relevant to this study. In addition to an introduction to the lymphocyte, this review will include a discussion of the current state of knowledge of lymphocyte activation, and a survey of the effects of other physical modalities (e.g., ionizing and nonionizing electromagnetic radiation, heat, osmotic shock) on lymphocyte physiology. While this dissertation is primarily concerned with mouse splenocytes, this review will, of necessity, include material from several other species and from cultured lymphoblasts.

As observed by light or transmission electromicroscopy (EM), the lymphocytes form a morphologically uniform class of small cells. They generally appear round and have a large nucleus occupying approximately 90% of the cell's diameter (124). Sizing of lymphocytes with a Coulter type transducer results in a positively skewed distribution of cells that would seem to coincide with the classical classification as small and large lymphocytes. For the mouse spleen this size distribution has a modal volume of 132.9 μm^3 (mean volume = 145.2 μ^3, skew = 0.528) which corresponds to a modal diameter of 6.33 μ (125,126). The small lymphocyte is present in far greater numbers in normal animals and is the cell generally associated with the name "lymphocyte."

Under the light microscope in stained smears or via phase contrast, this cell is observed as a round to oval nucleus surrounded by a thin halo of cytoplasm (121,123, 124,127). As indicated, there are also lymphocytes with larger amounts of cytoplasm making them intermediate in size between the small lymphocyte and the plasma cell. These cells have been called large lymphocytes or even broken into medium and large lymphocyte classes (124).

Unfortunately, these classifications proved to have little meaning and other distinctions were sought. The application of radioactive tracer methodology to the study of lymphocytes revealed that small lymphocytes formed at least two distinct populations: a short-lived population with life times on the order of hours to days and a long-lived population with life times of months to years (123). However, the most significant division of the lymphocyte population came with the recognition that there are two functionally distinct classes of lymphocytes, T-cells and B-cells (123,124,128). The T-, "thymus derived," or "thymus dependent" lymphocytes differentiate in the thymus, and are responsible for the so-called "cell mediated" immune phenomena (e.g. delayed hypersensitivity, transplantation immunity, cytotoxic reactions, and immunity to certain viral and fungus infections) (123,124,127,128). In addition, some T-lymphocytes develop into "helper" cells which interact

with B-cells to optimize antibody production (129). The B-, "bursa equivalent," or thymus "independent," lymphocytes[3] are responsible for the classical immune phenomena. On interacting with antigen, the B cells are activated to develop into "memory cells" or into plasma cells, producing and secreting large quantities of antibody (128-133). As indicated above, B- and T-lymphocytes are morphologically indistinguishable even with the aid of histo- and cytochemical techniques or electronmicroscopy (133,134). Differences in the surface morphology of B- and T-lymphocytes observed with a scanning electron microscope have been reported (135), but careful consideration has indicated that these differences are also inconsistent with respect to cell type (136). The best methods of distinguishing B- and T-lymphocytes utilize their surface coat characteristics (128,132-134,137,138). In the mouse, the most commonly used determinants are the theta antigen on T cells and the antibody coat (especially IgM) or the Fc or C_3 receptors characteristic

---

3. Historically, the existence of this functionally distinct class of lymphocytes was first demonstrated in the chicken where they differentiate in the bursa of Fabricius (130-132). This avian organ probably has no exact counter part in mammals where B-cell differentiation seems to take place in the bone marrow. This has given rise to the common practice of referring to B-lymphocytes as "bone marrow dependent cells."

for B-lymphocytes (138,139). The various T- and B-lymphocyte markers are summarized in Table 1. The mouse spleen has a mixed population of lymphocytes with approximately 35% T-cells, primarily in the periarteriolar areas, and about 40% B-cells, primarily in the white pulp (124,139).

The use of the surface coat to identify the two major classes of lymphocytes serves to emphasize the importance of this frequently overlooked structure. "The surface coat, or glycocalyx, is defined as the glycoprotein--and polysaccharide-rich coating, external to the plasma membrane, which is synthesized by the cell itself" (140). A surface coat of this type seems to be an important structural component of all mammalian cell types that have been studied (140,141). The surface coat is uniformly distributed over the cell surface with T-lymphocytes having a coat approximately twice as thick as B-lymphocytes (mouse: B ≃ 170 Å, T ≃ 290 Å) (140). The surface coat of lymphocytes contains a variety of carbohydrate moieties such as glycoproteins and glycolipids, RNA (15-30 μg/mg protein), and small amounts of DNA (0-10 μgm/mg protein), although the presence of DNA is still open to question. A cell's surface coat is of primary importance in the interaction of the cell with the environment. It is responsible for a major portion

TABLE 1

T- and B-lymphocyte Markers

	Name	Cell Type	Species of Occurrence	Notes
Cell surface marker	θ(Thy 1)	T	Mouse	High in thymus, low in peripheral T cells
	Ly(Ly-A)	T	Mouse	
	TL	T	Mouse	Present in thymus, usually absent in periphery
	MBLA	B	Mouse	
Cell surface immunoglobulins	IgG	B		5%
	IgM	B		The usual Ig on B-lymphocytes
	IgA	B		5%
	IgD	B		High in newborn
Response to mitogens in vitro	PHA	T	Mouse	B-cells respond in special circumstances
	Con A	T	Mouse	
	PWM	T & B	Mouse & Human	
	LPS	B	Mouse	
Rosetting Characteristics				
1. Heterologous erythrocytes (spontaneous)	SRFC	T	Human	
	MRFC	B	Human	
2. Fc receptors[a]	Fc Rosettes	B	Mouse & Human	
3. Complement receptors[b]	EAC Rosettes	B	Human & Mouse	
Scanning Electromicroscopy	"Hairiness"	B	Mouse & Human	Extensive microvilli of varying sizes
Surface Charge		T & B	Mouse & Human	The electrophoretic mobility of T-cells >B-cells, T-cells separate into at least two subclasses

[a]Antigen-antibody complexes of which the antigen is usually a heterologous erythrocyte.

[b]Antigen-antibody-complement complexes of which the antigen is usually a heterologous erythrocyte.

Adapted from Stathopoules and Davies (133)

of the cell's surface charge, the various receptor functions of the cell including cell-cell interactions, and the ability to recognize antigens, antibodies, mitogens, etc. (140,142,143). It has recently been suggested that the transduction mechanism associated with the binding of specific ligands to either the external or the internal surface of the cell membrane is the alteration of the molecular organization on the opposite surface of the membrane (104,142,144).

With the exception of the associated immunological determinants, relatively little is known about the composition of the lymphocyte plasma membrane. This is due in part to the difficulty in obtaining purified lymphocyte preparations, a problem that has been alleviated to a great extent by recent advances in cell separation technology (127). This and other technical problems and pitfalls associated with the study of mammalian cell membranes have recently been reviewed in general (145) and with special reference to lymphocyte membranes (128, 143). Despite these difficulties some information on the composition of lymphocyte membranes has become available from studies using lymphocytes from a variety of species (e.g. human, pig, rabbit, rat, calf thymus) and from cultured normal and leukemic lymphocytes (99,114,116,117). Lymphocyte membranes have approximately

equal amounts of lipid and protein, on a weight percent basis, and an additional 6%, by weight, carbohydrate (143). SDS-PAGE[4] of lymphocyte membrane proteins results in at least 30 polypeptide bands being stained for protein, of which about ten also stain positive for carbohydrate (128,143). The molecular weights of these different membrane proteins range from 1×10^4 to 2×10^5 (128,143). Of these groups of proteins, there are only four protein bands unique to B-cells and three bands that are unique to T-cells. Each lymphocyte is estimated to have about 10^7 protein molecules associated with its membrane, of these approximately 1.2×10^6 molecules are glycoproteins (143). Freeze fracture (em) studies indicate that there are relatively few intramembranous particles with inner and outer membrane particle densities of 350 and 100 μmm^{-2}, respectively. These studies also indicate that the particle distribution is different in B- and T-lymphocytes, with T-cell particles tending to form larger clusters (143). These data would seem to indicate that less than 1% of the lymphocyte membrane proteins actually transverse the membrane (143). Enzymes that have been found to be associated with the plasma

---

4. SDS-PAGE: Sodium dodecylsulphate-polyacrylamide gel electrophoresis.

membrane fraction isolated from lymphocytes disrupted and fractionated for biochemical studies include 5'-nucleotidase, Na/K-ATPase, acyl-coenzyme A:lysolecithin acyltransferases, and diglyceride kinase (128,143,146).

Normal resting lymphocytes have a membrane cholesterol: phospholipid molar ratio that is generally considered to be close to unity (reported values range from .42 to 1.03) (128,143,146). The phospholipids in lymphocyte membranes include phosphatidylcholine (PC) (≃ 40 mole %), phosphatidylethanolamine (PE) (≃ 30 mole %), phosphatidylserine (PS) plus phosphatidylinositol (PI) (≃ 10-12 mole %) sphingomyelin (≃ 10-14 mole %), and the remainder made up of phosphatidic acid and lysolecithin. Of particular note in this distribution of phospholipids is the relatively high concentration of sphingomyelin (i.e. 10-14 mole % compared with 5-6 mole % in the plasma membrane of liver cells), and the relatively low percentage of polyunsaturated fatty acids esterified to the PC and PE of resting lymphocyte membranes. The distribution of the fatty acids between positions 1 and 2 is also unusual with the unsaturated, oleic acid found to be preferentially esterified to position 1 while the content of the saturated fatty acid palmitic acid was high in position 2. It is also interesting that these lipid patterns are very consistent over at least six

different species indicating that the distribution of phospholipids and fatty acids are probably tissue specific (146).

The ionic constituents of lymphocytes are similar to those observed in the majority of mammalian cells. Values reported for the intracellular cation concentrations of mouse thymocytes and spleen cells suspended in Eagle's Medium were K+ = 103.6, Na^{+} = 31.9, Mg^{++} = 10.3, Ca^{++} = 0.9 mmoles/l ($\pm$ 5% variation) (147). This is a lower concentration of intracellular potassium than reported for rat thymus and spleen cells (148,149), normal and leukemic human lymphocytes (149,150), and cultured mouse lymphoblasts (151) all of which tend to be in the range of 131 mM-177 mM. The associated intracellular sodium concentrations of 14-32 mM were, however, in agreement with the values reported by Averdunk (147). The only anion concentrations that have been reported for lymphocytes are 43 mmole Cl^{-}/kg water for rat thymocytes (148) and only trace amounts of Cl^{-} in human lymphocytes (150). The reason for these discrepancies is not clear. It could be due to real interspecies differences, the variety of units used to report intracellular concentrations (mM, mmoles/l cells, mmole/l cell water, mmole/kg, ...), or experimental errors. It

is well established that accurate determinations of leukocyte monovalent ion concentrations and fluxes are difficult to make because of the very high rate of Na/K active transport in these cells (152-155). The water content of lymphocytes is reported to be in the range of 69-78.5% (147,149). The values reported for the intercellular concentration of calcium are slightly less than 1 mM (147,158), and are generally in good agreement despite the problems inherent in measuring the extremely small quantities of Ca^{++} contained in a cell (157,158).

Some new and interesting aspects of lymphocyte ion metabolism have recently come to light through the investigations of Negendank and Shaller (150,159-162). In a careful series of experiments these investigators studied the K^+-K^+, Na^+-Na^+, and Na^+-K^+ exchanges and intracellular concentrations of these cations in detail. The results from these investigations indicated that in human lymphocytes the intracellular potassium content exists as two components (150,159), and the intracellular sodium has three components (150,161). In addition, alterations in the K^+-Na^+ exchange rates were observed below 10°C with a critical temperature transition in the cell ionic composition centered at about 3°C (160). At 0°C in low sodium medium (19 mM Na^+, 5.4 mM K^+, isotonic

via sucrose) the intracellular concentration of Na^+ and K^+ were both maintained above their respective extracellular concentration (162). These results have been interpreted as adding support to Ling's "Association-Induction" hypothesis (150,163). There undoubtedly are other interpretations of these observations; never-the-less, it would seem that Association-Induction should be re-examined in light of these results (164,165).

Closely related to the ionic composition of lymphocytes are their electrical properties. It has been established that lymphocytes maintain an inside negative transmembrane potential similar to other mammalian cells. The resting potential of lymphocytes has been measured by microelectrode techniques to be between -7 mV (166) and -12 mV (167) or -15 to -18 mV (167,168) in PHA transformed cells. These values are considerably smaller than the -62 to -73 mV determined using the partitioning of radiolabeled or fluorescent lipophilic ions (166,169-171). This discrepancy is undoubtedly due to the various technical problems associated with these techniques. However, both techniques are in agreement in that stimulation with mitogen induces a rapid depolarization of the membrane followed by gradual repolarization over 7-12 hours (167,171).

The dielectric properties of the cultured mouse lymphoblasts (L5178Y) have also been determined from dielectric dispersion measurements over the frequency range 0.01-100 MHz. In order to obtain a good fit to the data a "double-shell" model had to be used. This was interpreted as indicating that both plasma and nuclear membranes, and nuclear and cytoplasm properties were being measured. On the basis of this model the following values were obtained: C_m = 1.0 $\mu F/cm^2$ and C_n ≃ 0.4 $\mu F/cm^2$ for the plasma and nuclear membrane specific capacities, ε_κ = 300, for the karyoplasmic permittivity, and κ_c/κ_a = 0.9, κ_k/κ_c ≃ 0.7 for the cytoplasmic/extracellular and karyoplasmic/cytoplasmic conductivity ratios, respectively (172).

Lymphocyte Activation

The vast majority of lymphocytes found in the circulation and lymphoid organs are true resting cells having basal levels of all metabolic processes (123). Consequently, the activation of lymphocytes by mitogen or antigenic stimulus is truly a case of inducing cells in a quiesent or G_o state to move out of that state into an active cell cycle, so the lymphocyte has recently become one of the preferred models for the study of cell activation, in general (173). Understanding the process of cell activation is one of the major problems in

biology today, and, unfortunately, the understanding of the processes leading to the initiation of proliferation in lymphocytes is no more advanced than the understanding of these processes in a more general context (173-175). This means that in studying cell activation in general or specifically with respect to lymphocytes, that one is faced with a large and growing body of information documenting a wide variety of cellular alterations believed to be associated with cell activation (174-179). Despite this vast number of observations, there is little understanding of the interaction of these changes and their relationship to the activation process as a whole (27,30,173,176,177,180). This lack of understanding of the inter-relationships of the various cellular processes involved in induction of proliferation greatly increases the difficulties inherent in establishing the necessary and sufficient conditions for initiating a process that is temporally separated from the initiating event by two or three days (156). This situation may be further obscured if there is, in fact, no single sufficient condition for the initiation of the activation process, but instead a group of necessary conditions with some requisite temporal organization. A situation that is not unlikely. The magnitude of this problem can be illustrated by a brief survey of the

various cellular changes that have been observed following mitogenic or antigenic stimulation of lymphocytes (27,127,176, 177).

It has been established that the external signal and the initial step in the stimulation of lymphocytes is the binding of the mitogenic or antigenic agent to a receptor on the cell surface, or some other perturbation of the cell surface (Table 2) (127,178,180). Stimulation requires multivalent agents capable of cross-linking to form "patches" on the cell surface. Patch formation is a nonenergetic phenomena which, at 37°C, is frequently followed by the energy-requiring process of capping (181,182). The initial clustering of receptor-mitogen (or antigen) complexes seems to be an integral part of the transduction process; however, the lateral movement of these patches toward one pole of the cell where they are collected to form a "cap" does not appear to be necessary for lymphocyte activation (181-184). The capping process has received much attention, and seems to end in either pinocytosis of the cap or shedding of the cap material into the medium (181,182). The best evidence that the mitogenic stimulation is strictly an extracellular process is the fact that Con-A or PHA remain effective lymphocyte stimulators even after being convalently linked to a plastic surface or to polyacrylamide or Sephadex beads (127). It is important to

TABLE 2A

Mitogenic Agents Used to Stimulate In Vitro Lymphocyte Proliferation

	Mitogens	Specificity	Comments
1.	Seed Extracts		
	Phytohemagglutinin (PHA)	T	agglutinate cells, maximum mitogenic activity achieved at 3-10% of cells binding capacity, competitively inhibited by N-acetyl-D-glucosamine branched galactose containing disaccarides
	Concanavalan A (Con-A)	T	agglutinate cells, maximum mitogenic activity achieved at 3-10% of cells binding capacity, competitively inhibited by α-methyl-D-mannoside and α-methyl-D-glucopyranoside
	Pokeweed (PWM)	T & B	
2.	Divalent Cations		
	Hg^{++}		Binds to SH groups
	Zn^{++}		Prosthetic group on certain enzymes
	Ni^{++}		
3.	Bacterial Products		
	Streptolysin 10	T & B	
	Lipopolysaccardies (LPS)		
	E. coli endotoxin	B	Lipid A moiety is active component
	Staphylococcal endotoxin B	T	

(Continued)

TABLE 2B

Mitogenic Agents Used to Stimulate In Vitro Lymphocyte Proliferation

(Continued)

	Mitogens	Specificity	Comments
4.	Antibodies		Prolonged exposure required
	Anti-leukocyte sera		Against cell surface components
	Anti-immunoglobulin sera		B cells respond
5.	Others		
	Trypsin	B	Inhibited by proteolytic inhibitor
	Chymotrypsin		
	Papain		
	Periodate		Partial inhibition by borohydrate reduction
	Neurominidase-galactose oxidase		Partial inhibition by borohydrate reduction
	Mixtures of leukocytes (MLR)		Interaction of surface components
	Tuberculin P.P.D.		Requires previous antigenic exposure
	Tetanus toxoid		
	Diphtheria toxin		
	Pollen extracts		
	Keyhole limpet hemocyanin		
	Ca^{++} ionophore, A-23187		

Adapted from Loeb (178)

realize, however, that there is no correspondence between the ability of a lymphocyte to bind a given lectin and the stimulatory activity of that lectin toward that lymphocyte. It is well established that a number of nonmitogenic lectins bind to lymphocytes, and that both B and T cells bind Con-A, PHA and LPS even though Con-A and PHA are exclusively T cell mitogens while LPS stimulates only B cells (127,178,180).

In the case of B-lymphocyte stimulation by LPS the situation is even more complex since it is believed that the active part of the lipopolysaccharide molecule is the lipid moiety, Lipid A, which can intercollate directly into the membrane lipid (185). This may require a change in the point of view that the transmembrane signal-transducing receptors are glycoproteins; however, it is compatible with the recently proposed interactive model of B-cell activation (186,187). This three-state allosteric model provides a general, quantitative approach to B-cell activation based on the concept of an allosteric, transmembrane, receptor-transducer molecule or molecular complex (186). This model is more complete than previous models, which can be viewed as special cases of this model, and is able to account for the wide variety of B-lymphocyte immunologic responses. This model is an exciting proposal; however, it also serves

to illustrate one aspect of the problem of gaining insight into the activation process; that is, while accounting for the immunological properties of B cells this model gives no indication as to the subcellular events required to initiate the activating process (186,187).

Several other aspects of this problem are illustrated by considering the various alterations of ionic fluxes reported to occur within minutes of cellular binding of mitogen or antigen. In particular, these reports illustrate the extreme difficulty of establishing cause-effect relationships between temporally distant events (156), and the difficulties created by the failure to consider the inter-relationships between various intracellular events (27,155,156). The potential ability of changes in monovalent cation fluxes to serve as regulators of cell growth and proliferation has received considerable attention recently (27-34). Despite great enthusiasm in this area (31,32,188), careful examination of the data indicates that the case for K^+ and/or Na^+ control of lymphocyte activation is far from convincing (27). Unfortunately, much of the published data on alterations in potassium and sodium transport in mitogen-treated lymphocytes is of very limited value (27) due to incomplete experimental designs and the failure of most

investigators to fully appreciate the difficulties associated with the accurate measurement of ion fluxes and intracellular ion concentrations (154,155). These problems have been discussed in detail in a review by Lauf (27). The problems include the failure to monitor changes in cell volume, the use of inappropriate concentrations of mitogen, oubain, or other drugs, and the limited scope of the studies. The scope of an investigation is particularly important to the interpretation of the results, and it is unfortunate that the majority of studies on mitogen-induced changes in ion fluxes have been limited to PHA or Con-A (31,32). The value of a more complete protocol is illustrated by the findings of Averdunk (147) who included a few experiments with PHA, LPS, and PWM in addition to his main investigation with Con-A. This study also included measurements of Mg^{++} and Ca^{++} in addition to Na^{+} and K^{+} fluxes in both mouse thymocytes (almost pure T cells) and mouse spleen cells (mixed B and T cells). This comprehensive protocol lead to the unexpected observation that the mitogen-induced increases in membrane permeability were the same in both cell populations regardless of the mitogen (Con-A and PHA are only mitogen for T cells, LPS is only a B cell mitogen and PWM is mitogenic to both types of lymphocytes). This observation leads to the conclusion that

TABLE 3A

Sequence of Molecular Events During Lymphocyte Transformation

I. Early Events (within 2 hr)

A. Increases in Membrane Associated Events

1. Phospholipid turnover (increased polyunsaturation)
2. Choline Incorporation into lipids
3. Amino acid transport
4. Facilitated diffusion of 3-0 methylglucose
5. ^{42}K uptake
6. $^{45}Ca^{2+}$ uptake

B. Increases in Nuclear Associated Events

1. Acetate incorporation into histones
2. Phosphorylation of histones and nuclear proteins
3. Binding of acridine orange to chromatin
4. Rate of RNA synthesis, 4S, 45S and polydisperse
5. RNA

C. Cytoplasmic Associated Events

1. Increase glucose utilization
2. Increase glycolysis
3. Increase consumption of oxygen
4. Redistribution of lysosomal enzymes

Adapted from Loeb (178)

TABLE 3B

Sequence of Molecular Events During
Lymphocyte Transformation

(Continued)

II. Intermediate Events (2 to 14 hr)

A. Related to RNA Synthesis

1. Increase in uridine kinase activity
2. Increase DNA-dependent RNA polymerase A activity
3. Increased synthesis of ribosomal RNA
4. Reduced loss of 18S ribosomal RNA

B. Related to Protein Synthesis

1. Increased amino acid incorporation into protein
2. Increased proportion of ribosomes synthesizing proteins

C. Other Metabolic Changes

1. Increased incorporation of glucosamine into membrane
2. Increased activity of S-adenosyl methionine decarboxylase
3. Increased activity of ornithine decarboxylase

Adapted from Loeb (178)

TABLE 3C

Sequence of Molecular Events During
Lymphocyte Transformation

(Continued)

III. Late Events (16 to 72 hr)

A. Increased in Enzyme Activity Related to DNA Synthesis

1. DNA polymerase activity (30-200-fold)
2. Thymidine kinase activity (2-10-fold)
3. Thymidylate kinase activity (2-10-fold)
4. Deoxycytidine kinase activity
5. Deoxycytidylate deamine activity

B. Events Reflecting DNA Replication

1. Incorporation of thymidine into DNA
2. Incorporation of ^{32}P into DNA
3. Increased DNA content per culture

C. Changes in Activity of Other Enzymes

1. Alteration in isozyme profile of lactic dehydrogenase
2. Increased activity of glucose-6 phosphatase
3. Increased activity of α and β-glucosidase

Adapted from Loeb (178)

the observed permeability changes are associated with the binding of the mitogen to the cell surface, but are not necessarily a trigger of the activation process (147), a conclusion similar to that reached by Negendunk and Collier (189).

The case for Ca^{++} functioning as a trigger in the activation of lymphocytes is only slightly stronger (156) despite the ubiquitous role of Ca^{++} and associated cyclic nucleotides as mediators of a wide variety of cell processes. These studies have been particularly difficult due to the extremly small quantities of calcium found in cells (157,158). However, there is some evidence that Ca^{++} is required for the proliferation of lymphocytes (190), and Ca^{++} ionophor has been shown to induce the transformation of lymphocytes (191). The increase of intracellular Ca^{++} is one of the earliest events observed following the addition of mitogen to a suspension of lymphocytes (192-196), and the Con-A-induced increase in calcium influx is reported to be specific to T cells (194). This is strong, but not conclusive, evidence in favor of a calcium hypothesis (156).

Physical Perturbation of Lymphocytes

Of the various physical modalities that have been investigated with respect to their effects on the structure

and function of lymphocytes, the effects of ionizing and nonionizing electromagnetic radiation, hyperthermia, ultrasound and osmotic shock are particularly germane to this study.

One of the most important functions of the cell membrane is to provide a barrier enabling the cell to maintain the homeostatic intracellular environment required by the various physiological processes needed to sustain the cell's functional integrity. Consequently, the response of a cell to osmotic stress is one of the oldest methods used to investigate the structure and behavior of the plasma membrane (197), and the response of a cell to osmotic stress provides a convenient monitor of that cell's physiological status (198). Bauer et al. (197) investigated cell membrane damage caused by the exposure of rat thymocytes to media of different hypotonic osmolarities at 37° and 4°C for a period of 5 minutes. Alterations in the membrane were monitored by measuring the permeation of K^+, fluoresein, and lactate dehydrogenase (LDH), and by freeze-etch electron microscopy. The release of the biochemical markers was a function of the molecular weight of the marker and the osmolarity of the medium, with larger molecules being released to a greater extent as the osmolarity of the medium decreased. The cells were more sensitive to

osmotic stress at 4°C than they were at 37°C and the freeze-etched electron micrographs indicated that increasing hypotonicity resulted in decreases in the number of intramembrane particles together with increasing aggregation of the remaining particles. It is interesting to note that potassium release was the most sensitive indicator of membrane damage; while Trypan blue dye exclusion was found to be too insensitive to small osmolytic changes and too prone to give erroneous readings to be useful in this study (197).

The results of a more extensive series of experiments on the effects of osmotic stress on cultured mouse lymphoblasts (L5178-Y) have recently been reported by Buckhold Shank and colleagues (151,198-200). This investigation was designed to study the time course and mechanisms of volume regulation in nucleated mammalian cells. The results of this study revealed that these cells and several other culture lines and tumor cells respond much faster to a hypotonic shock than do red blood cells. Upon dilution of the culture medium with an equal volume of water, the cells immediately swell as water and Na^+ diffuse into, and K^+ diffuses out of the cell. This initial phase lasts about 2 minutes at which time the cell volume and intracellular sodium concentration

have increased to peak values 1.4 and 2.7 times normal, respectively, and the intracellular potassium concentration has decreased to about half its normal value. At this point, the cells begin volume regulation by actively transporting sodium out of the cell causing a shift in the osmotic gradient causing water to diffuse out of the cell. This process continues until the cell volume has returned to its normal value after 20 minutes (151,199). This volume regulation process is interesting in that the intracellular K^+ concentration remains at 1/2 its normal level for at least the first 15 minutes of the recovery process. At that time, the rate of change of the cell volume and intra-cellular Na^+ concentration have slowed as they approach normal values, and only then does potassium begin to be exchanged for sodium. The net result is that on shrinking to its normal volume the cell has a normal or slightly elevated intracellular Na^+ concentration and decreased K^+ and anion concentrations. The volume regulation process is oubain-sensitive and dependent on the presence of Na^+ and K^+ in the medium (K^+ can be replaced by Rb^+ or Cs^+ but Na^+ has no substitute) (151,195). This high rate of volume regulation in hypotonic media was observed in a variety of nucleated mammalian cells, and differs only in the amplitude of the initial swelling over a wide

range of hypotonicities (30-75% of iso-osmotic) (199, 200). When the osmolarity of the test medium approaches 25% of isotonicity or lower, the time course of volume regulation begins to increase (199); however, it is at this point that the viability of the cells begin to be effected (199,201). The dependence of this volume control process in lymphocytes on the ATP-dependent efflux of Na^+ has led to the suggestion that the time course of this reaction to osmotic shock could be used as an indirect monitor of intracellular ATP levels (198).

The small lymphocyte is one of the most radiosensitive mammalian cells, both in vivo and in vitro (202). These small quiesent cells have been shown to be effected by an in vitro exposure to as little as two roentgens of x-radiation (203). This extreme sensitivity to ionizing radiation is greatly reduced by mitogenic or antigenic stimulation, even when the activating stimulus follows the irradiation of the cells (204-209). The reasons for lymphocytes' extreme radiosensitivity and its potentiation by activating stimuli have been the object of much speculation and many experimental investigations. One plausable explanation for these phenomena is the so-called "Round Cell Theory" which attributes the high radiosensitivity of small resting cells with

high nuclear: cytoplasmic ratios, such as lymphocytes, to the basal metabolic levels maintained in these cells and the resultant lack of subcellular components needed for the repair of subcellular lesions. By extrapolation, the protective effects of mitogen or antigen activation of lymphocytes is due to the stimulation of the cells' synthetic processes. This theory is supported by experimental data, and has some qualitative predictive value.

A more specific, though compatible, theory explaining the high radiosensitivity of the lymphocyte can be based on the observed changes in the functioning of the plasma membrane of irradiated cells (204-211). The loss of intracellular K^+ into the suspending medium has been established as the most sensitive indicator of radiation-induced cell injury (208). In addition to this and other observations of a radiation produced increase in the permeability of the cell membrane to potassium and other soluble materials (205-211), Sato, et al. (206) have observed a dose-dependent reduction in the electrophoretic mobility of irradiated rat thymocytes (206). The treatment of the cells with PHA produces a slight increase in the electrophoretic mobility of the thymocytes and prevents the marked reduction produced by x-irradiation. The combined PHA-X-ray treatment produces a mobility between the untreated

control value and the slightly elevated PHA-treated mobility (206). In contrast, studies of the effects of *in vitro* irradiation on the rosette formation of human lymphocytes with sheep red blood cells indicate that ionizing irradiation produces little (204) or no change (211) in the ability of lymphocytes, to form E or HEAC rosettes. The significance of the cell membrane in the reduction of lymphocyte radiosensitivities by PHA stimulation is illustrated by the recent study of carrier-mediated nucleotide transport in irradiated lymphocytes with and without PHA stimulation (206). Unstimulated lymphocytes were unable to transport thymidine into the cell for repair of DNA damage, while PHA-stimulated cells activated this transport system at the G_1-S interface permitting cells irradiated in S phase to undergo considerable repair activity, as indicated by incorporation of TCA insoluble ^{3}H-thymidine (206). A depression of this thymidine transport system was observed at doses greater than 10 Gy (206).

A number of investigations have been reported on the effects of ionizing radiation on the response of lymphocytes to mitogen stimulation and allogenic stimulation in mixed leukocyte cultures (MLC) (204, 212-214). There is good agreement between the various investigations indicating that there is a dose-related decrease in the

response of irradiated lymphocytes to activating stimuli, and this decrease in activity relative to unirradiated controls increases with the length of time between stimulation and the measurement of the response. This decreased level of response is related to the combined effects of interphase death and dose-dependent delays in G_2 and late G_1 (213). PHA stimulation has been observed to be more radioresistant than stimulation by PPD or the MLC response (212-214). The relative sensitivity of B and T cells has been investigated using lymphocyte transformation (203,211-213) and the in vivo migration of irradiated cells (214). These two techniques give conflicting results in that in vivo migration indicates that B cells have the greater radiosensitivity (215), while the mitogen cultures imply that T cells are the more sensitive cells (204). This discrepancy can probably be resolved by a better experimental protocol incorporating either purified cell preparation or both T and B cell specific mitogens.

The lymphocyte may also prove to be very sensitive to nonionizing electromagnetic radiation, particularly in the microwave (MW) and radiofrequency (RF) regions of the spectrum; however, there are still too few studies to decide this issue (8,11). At the present time, there are a number of on-going investigations into the effects

of MW and RF radiation on lymphocytes (11). This current high level of research activity is due in part to the resurgent interest in nonionizing radiation plus the stimulus of several reports of changes in lymphocyte functions following in vivo (216-223) and in vitro (223,224) exposure to MW and RF radiation. Various changes in the immune systems of monkeys, rabbits, rats, mice, and guinea pigs have been observed following exposure to MW and RF under a variety of exposure conditions. These changes include shifts in the circadian rhythms of the hemopoietic stem cells (8,216), increases in antibody-producing cells in the circulation and lymphoid organs (216-218), increases in serum antibody titers (216,222), an increase in the mitotic index of spleen lymphocytes, and chromosomal disorganization and other mitotic abnormalities in cells from the lymphoid organs (8,218-220). Many of these phenomena are transient in nature, appearing about three days post-irradiation then returning to baseline levels in three to six days.

One of the more extensively studied of these changes is the observation that a single 30 minute exposure of CBA/J mice to 2.45 GHz microwave radiation (0.7 mW/cm^2) is followed three days later, by a transient increase in the number of spleen cells bearing receptors for the third component of complement (CR^+) (225). At higher

power levels or longer exposures this is accompanied by increases in the number of Fc receptor positive cells (FcR^+) and the number of cells bearing surface immunoglobulins (sIg) (225,226). The threshold for increasing the number of CR^+ cells has been established to be 10 J/gm of body weight in the mouse. This can be given in a single dose or multiple exposures given at intervals of one hour or less (227). This response to MW irradiation is specific for mice with the haplotype $H2^k$ (227), is probably due to the induction of existing B cells to undergo maturation (228), and may involve a soluble humoral factor (230).

One of the more interesting effects reported to result from the MW or RF irradiation of lymphocytes is the observation that a blastogenic transformation could be induced by the in vitro exposure of human lymphocytes to pulsed 10 cm microwaves at 7 mW/cm^2 for 4 hours per day or 20 mW/cm^2 for 15 minutes per day on each of the 3 or 5 days used to culture the cells (22). This very interesting observation has proven to be very difficult to confirm (231). There have also been several reports of an enhanced response to mitogenic stimulation by lymphocytes exposed to nonionizing radiation (11,222, 232). In one recent investigation rats were subjected to chronic, low-level microwave exposure (2.45 GHz,

5mW/cm^2, 4 hr/day) beginning *in utero* on day 6 of gestation and extending to day 20 or day 40 postpartum. Spleen cells from these rats showed an enhanced response to both T and B cell mitogens (PHA and LPS) relative to lymphocytes from sham-irradiated controls (232).

The lymphocyte has also been the object of research on the effects of hyperthermia. *In vivo* hyperthermia including that associated with acute thermal injury has been reported to result in the release of a cytotoxic factor (233) and the induction of suppressor T cell activity (234). More detailed studies on lymphocytes exposed to elevated temperatures have been carried out taking advantage of the more carefully controlled conditions afforded by *in vitro* experiments. Exposure to a temperature of 43°C has been shown to result in a time dependent loss of cytolytic activity and colony-forming ability of T lymphocytes (235,236). It was noted, however, that the loss of colony-forming ability required exposure of these cells to hyperthermic conditions for a period four times as long as that needed to produce a comparable loss in cytolytic activity (236). This result suggests the possibility of repair processes acting to repair potentially lethal and sub-lethal damage (236). This idea is supported by a more detailed investigation into the effects of hyperthermia on T-lymphocyte primary and secondary cytolytic activity

(237). These studies clearly indicate that the effects of hyperthermia on the immunocompetence of lymphocytes is a more complex phenomenon than it was originally believed to be (236,237). The complexity of hyperthermic injury in lymphocytes is born out by the morphological studies of Schrek and his colleagues (238-240). Using an inverted microscope equipped with a high power (100X), oil immersion, phase contrast objective, Schrek has observed suspensions of lymphocytes at temperatures from 37° to 50°C (238). As a result of these studies he has been able to identify at least two morphologically distinct modes of hyperthermic death in lymphocytes, depending on the temperature. Lymphocytes at temperatures of 37°, 41° or 42°C die primarily by pyknosis, and lymphocytes at 43° or 45°C die from processes resulting in the formation of "condensed cells" (240). These observations have recently been confirmed by electron micrographic studies (240).

Finally, there are the very interesting observations of Chapman et al. (241) on the effects of ultrasound on rat thymocytes. These cells were suspended in glucose supplemented Krebs-Ringer solution with added calf serum (1:1 or 1:3) and subjected to various ultrasonic field conditions after which they were monitored for cell survival on the basis of cell lysis and Erythrosin B dye

exclusion. Sonication at an intensity of $1W/cm^2$ for 10 minutes at frequencies of 0.75, 1.0, 1.5, and 3.0 MHz resulted in cell survivals of about 20%, 70% 85%, and 99+%, respectively, relative to sham exposed controls. Exposure of lymphocytes to an ultrasonic field of $2W/cm^2$ for 40 minutes at 3.0 MHz still failed to produce any detectible difference in the viability of experimental and control cells. Additional experiments at 1.5 and 3.0 MHz confirmed the observation of an intensity dependent decrease in cell viability at 1.5 MHz and no change at 3.0 MHz. Interestingly, determinations of intracellular K^+ concentrations indicated an intensity dependent decrease in intracellular K^+ at 3.0 MHz that was about twice that at 1.5 MHz at any given field intensity. Isotopic studies indicated that the decrease in intracellular K^+ at 3.0 MHz was the net result of a decreased K^+ influx and increased K^+ efflux. The authors suggested microstreaming as the mechanism responsible for the membrane damage at 3.0 MHz (241).

MATERIALS AND METHODS

A schematic diagram of the general protocol used in this investigation is presented in Figure 1. Details concerning the various steps are provided below.

Unless otherwise noted, the chemicals used in this study were obtained through usual commercial sources, and were used as they were received without further purifications.

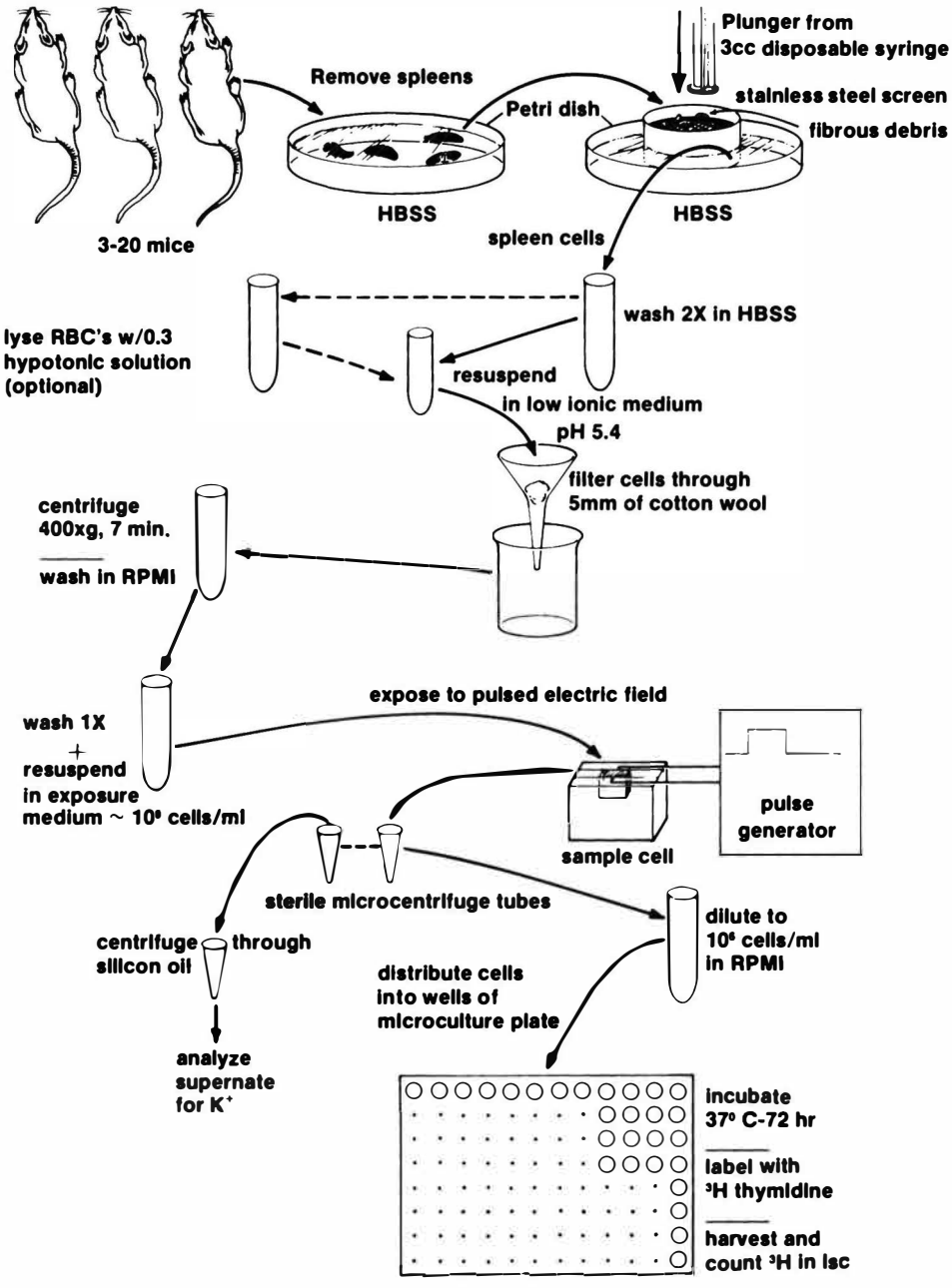
The Solutions

All of the solutions used in this study were tested and adjusted to be iso-osmotic with mouse serum, 308 $\pm$ 2 mOsm (242-244).[5] The osmotic pressure of these solutions was measured with a freezing point depression osmometer (Advanced Instruments, Model L5/3), and when necessary a solution's osmotic pressure was adjusted by the addition of an appropriate amount of deionized water, 1 m NaCl, or 1 m Sucrose. Hanks' balanced salt solution (HBSS) was obtained (without pH indicator) as a 10X solution (GIBCO). This stock solution was subsequently diluted with the appropriate amounts of deionized

---

5. The exception to this practice of adjusting solution osmolalities to 308 mOsm was the tissue culture medium which was used as it was obtained from the intralaboratory pool in order to avoid additional problems of sterility.

Figure 1: Schematic diagram of experimental protocol for this study, for details see text.


Plunger from
3cc disposable syringe
Remove spleens
Petri dish
stainless steel screen
fibrous debris
HBSS
HBSS
3-20 mice
spleen cells
wash 2X in HBSS
lyse RBC's w/0.3
hypotonic solution
(optional)
resuspend
in low ionic medium
pH 5.4
filter cells through
5mm of cotton wool
centrifuge
400xg, 7 min.
wash in RPMI
wash 1X
+
resuspend
in exposure
medium ~ 10⁸ cells/ml
expose to pulsed electric field
pulse
generator
sample cell
sterile microcentrifuge tubes
centrifuge through
silicon oil
dilute to
10⁶ cells/ml
in RPMI
distribute cells
into wells of
microculture plate
analyze
supernate
for K⁺
incubate
37° C-72 hr
label with
³H thymidine
harvest and
count ³H in lsc

water and 7.5% sodium bicarbonate solution (GIBCO), and supplemented with 25 mM HEPES buffer. This was adjusted to pH 7.4 at room temperature (-22°C), and to 308 mOsm, as indicated above. Iso-osmotic sucrose solution (308 mOsm) was made with density gradient grade sucrose (Swartz-Mann). The exposure medium was made from this iso-osmotic sucrose solution and HBSS with volume ratios from 64:30 to 75:25, the ratio being selected to give the desired impedence for the cell suspension in the exposure cell (i.e. 350-400 Ω at 1 kHz).

The tissue culture medium used was RPMI-1640 (GIBCO) supplemented with L-glutamine (200 mM), 25 mM HEPES buffer, pooled normal human plasma to 10%, penicillin (100 units/ml), streptomycin (100 mg/ml), in later experiments gentamycin (50 mg/l). Hereafter, this complete medium is referred to simply as RPMI.

The plasma used throughout this study was reclaimed plasma obtained after preparing packed cells from units of whole blood.[6] The blood was originally obtained from normal healty human volunteers, and was tested hepatitis free. The plasma was obtained in individual plastic blood storage bags, and was stored at 4°C during the

---

6. I wish to thank Dr. John L. Thorton of the Richmond Metropolitan Blood Service for providing this plasma.

initial screening. Each individual unit was tested for sterility by incubating 1 ml of plasma on a blood agar plate for 24 hours. This was followed by testing each unit for the ability to support lymphocyte blastogenesis using the microculture technique described below. Those units showing lymphocyte support activity (6 of 11) were then pooled, divided into 100 ml aliquots, and stored at -20°C until used.

The Cells

The majority of the cells used in this study were lymphocytes obtained from mouse spleens using standard procedures (244,252). Inbred mice (C57BL/6 or C3H) of either sex, obtained from various commercial vendors, were used throughout the study; although, females were used in at least 90% of the experiments. All of the mice used were between 7 and 40 weeks old, with the majority being in the 12-14 week range. This variation in age did not seem to effect the results in any systematic way. The mice were sacrificed via chloroform, and their spleens removed asceptically and placed in HBSS. The spleens were cut into pieces with small dissecting scissors the cells were teased out of the spleen capsule through a course stainless steel screen into HBSS with the plunger from a 3 cc disposable plastic syringe. The

number of spleens being teased through a screen at any one time was kept below five to insure a good cell yield (generally 5-10 x 10^7 viable mononuclear cells were recovered for each mouse used). These cells were then pooled (up to 20 spleens were used in the larger experiments), and washed twice with HBSS to remove small debris. The cell pellet was then resuspended in 10-15 ml low ionic media (LIM). This suspension was then filtered through 5 mm of loosely packed cotton wool to remove dead and damaged cells (245). The low ionic solution used here was sucrose in 0.05 M citrate buffer, pH 5.4, adjusted to 308 mOsm. This seemed to give better results in our hands than sorbitol, suggested by Von Boehmer and Shortman (245). The filtered suspension was then centrifuged 200 x g for 10 minutes, the resulting cell pellet washed once in 40-50 ml of RPMI followed by one wash in 10 ml of exposure medium. During the centrifugation step of this last wash the cells were counted by hemocytometer, and their viability checked by trypan blue dye exclusion. The cell pellet from the exposure medium wash was then resuspended in the appropriate amount of exposure medium to give the cell concentration desired for that particular experiment (generally 10^8 cells per ml).

In those experiments where the presence of a large number of erythrocytes would effect the results (e.g. studies of Na/K fluxes) the above procedure was altered by using only a single initial HBSS wash followed by resuspending the cells in a small volume of HBSS. Enough deionized water was added to lower the tonicity of the suspension to 0.3 of isotonic. After 45-60 seconds the suspension is brought back to isotonicity with 10 x HBSS, RPMI was added to bring the volume to 14 ml, and the suspension centrifuged (200 x g, 10 min). During this centrifugation samples of the suspension are examined microscopically to determine the size of the surviving erythrocyte population. If the number of RBCs remaining was still too large the procedure was repeated, otherwise the suspension was returned to the LIM filtration step in the standard procedure. This method of RBC lysis is based on the work of Thomson et al. (201) who studied lymphocyte susceptibility to hypotonic shock. Their investigation indicates that using 0.3 isotonic solution should minimize the lymphocyte loss in the initial exposure to hypotonicity, and almost eliminates such losses on subsequent encounters with hypotonicity (201).

In addition to mouse spleen lymphocytes, a few exploratory experiments have been performed during the

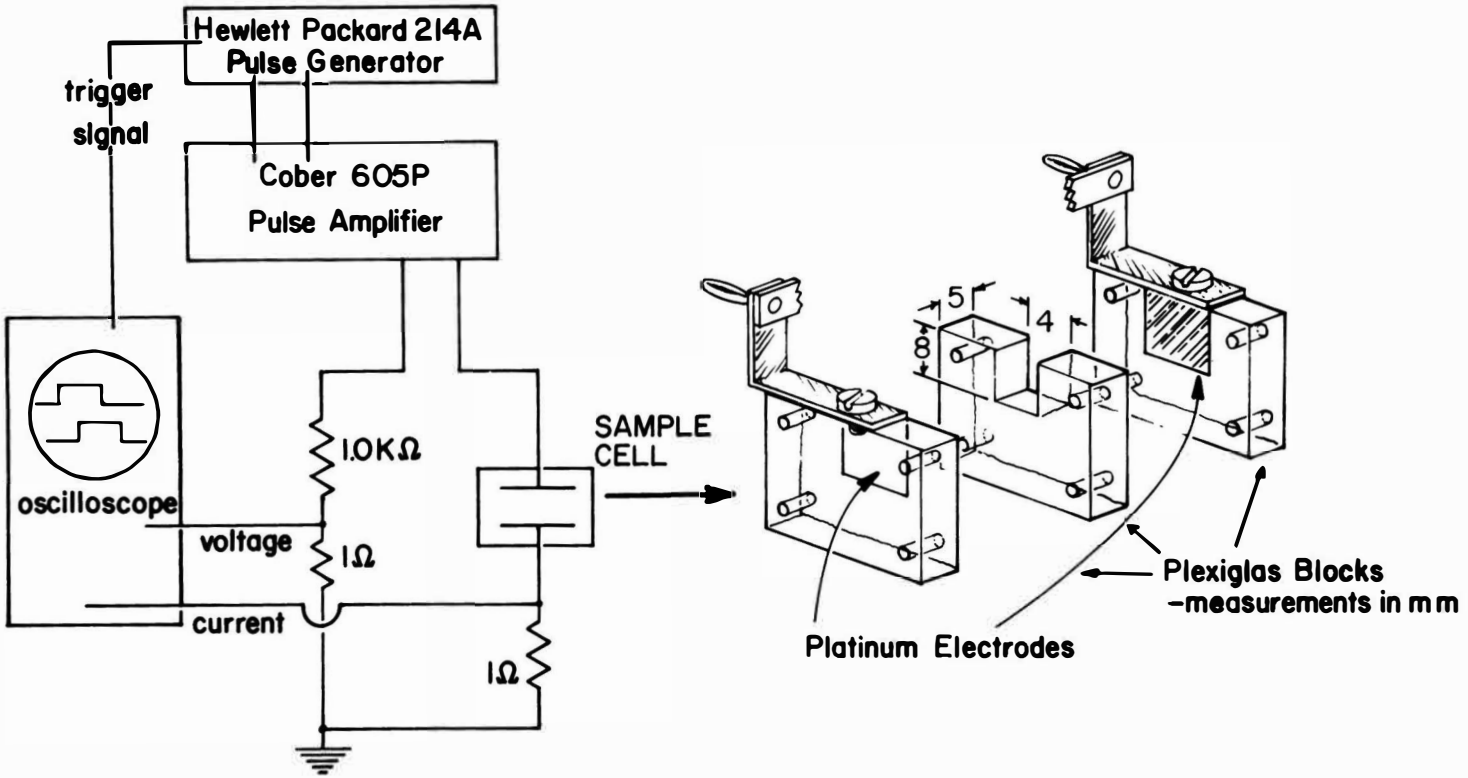
course of this investigation using Ehrlich acites tumor cells. These cells were harvested by flushing the peritoneal cavity of a mouse with HBSS or saline 5-15 days after injecting the mouse with 10^6 Ehrlich tumor cells, i.p. The cells were washed twice with HBSS and once with exposure medium before finally resuspending the cells in exposure medium (10^8 cells/ml). These cells are extremely metabolically active and were kept at 0°C in an ice water slush before and after exposure to the pulsed field. Ehrlich ascites tumor cells are very prolific so the ascites from a single mouse can yield 10^8-10^9 cells.

Both lymphocytes and Ehrlich ascites cells were exposed in suspensions of 5×10^7 - 10^8 cells per ml with a sample size of 140 µl per exposure. In some cases duplicate samples were exposed sequentially and pooled to insure an adequate number of cells for the planned analyses.

The Exposure System

The system used to expose cells to a high field strength pulsed DC electric field (Fig. 2) is built around the pulse generating system. This pulse generating system is made up of a Hewlett Packard Model 214A pulse generator and a Cober Electronics model 605P pulse amplifier. This combination of instruments produces a

Figure 2: Schematic diagram of pulse generating and monitoring circuit including an exploded view of the sample cell (insert).


Hewlett Packard 214A
Pulse Generator
trigger
signal
Cober 605P
Pulse Amplifier
oscilloscope
voltage
current
1.0KΩ
1Ω
1Ω
SAMPLE
CELL
5
4
8
Plexiglas Blocks
-measurements in mm
Platinum Electrodes

square wave pulse with rise and fall times of 20 and 35 nsec, respectively, and pulse widths from 50 nsec to 1 msec. The pulse amplitude is continuously variable up to 2200 volts at 11 amperes into a 200 Ω load. These pulses can be produced as a single shot or repetitively up to 1 MHz. The pulse width and the voltage and current amplitudes of the pulses were monitored using a resistive voltage divider similar to that described by Kinosita and Tsong (5). This monitoring circuit was constructed using precision (1%) noninductive high voltage metal film resistors.[7] The output from this monitor was recorded using either a Tektronics type 454, 125 MHz oscilloscope equipped with a Tektronics type C-31 oscilloscope camera, or a Tektronics type 556 Dual Beam oscilloscope with one type 1A1 dual trace plug-in unit (50 MHz) and one type CA dual trace plug-in unit (24 MHz) and a type C-27 oscilloscope camera. The former unit was used for the initial experiments while the latter oscilloscope was used for the main body of experiments. Both cameras were equipped with Polaroid roll-type camera back, and the pulse traces were recorded with Polaroid type 410 oscillographic film (ASA 10,000).

---

7. Craddock Electronics, Inc., 3127 Chicago Avenue, Riverside, California, 92507.

Cells were exposed to pulsed electric fields in a plexiglass chamber having inside dimensions of 5 mm x 4 mm x 8 mm. The electrodes were made from platinum foil attached to the plexiglass to form the sides of the exposure chamber (Fig. 2, insert), making the inter-electrode distance 5 mm. The exposure chamber was connected directly to the pulse monitor by two rigidly mounted banana plugs. In some experiments the sample temperature was measured immediately before and immediately after the exposure using a fast thermocouple probe (Baily time constant .025 msec). Before exposure, the impedence of each sample is determined at 1 kHz using a General Radio type 1650-A Impedence Bridge.

The Analysis of Effects

Alterations of membrane permeability were monitored by measuring sodium and potassium concentrations in the suspending medium by flame photometry (IL Model 434) using a 15 mM LiCl internal standard. Following exposure to an electrical pulse the 140 µl cell suspension was layered over silicon oil (G.E. SF 1230)[8] in a 500 µl polyethylene microcentrifuge tube, and set aside. After 50-70 minutes the microcentrifuge tubes were

8. Courtesy of James Frewin, Silicon Products Department, General Electric Company, Waterford, New York, 12188.

placed in a Beckman Model B "Microfuge" and spun at 11,000 x g for 45 seconds. The cells (ρ = 1.077 gm/ml) are centrifuged through the silicon oil (ρ = 1.050 gm/ml) leaving the suspending solution on top of the silicon oil layer. This technique enables one to obtain a rapid and complete separation of medium and cells thus ending any exchange of constituents between the two (246). This technique provides the experimental convenience of allowing the solution to be removed at some convenient time rather than immediately following centrifugation, and still allowing the cells in each sample to be in suspension for approximately the same length of time (60 min) following exposure to the pulsed field. After all of the samples have been exposed to the electric field and centrifuged, the solution was removed from the silicon layer and four 10 μl aliquots are taken and diluted, 1 in 200, with 15 mEq/l LiCl. The values obtained were then compared with sham exposed controls and with samples that had been sonicated to obtain complete disruption of the cells for a maximum release value.

In some experiments 20 μl aliquots of cell suspension were removed from the sample before centrifugation through the silicon oil to obtain accurate cell counts using a Coulter counter (Model ZH), and to measure cell

volume changes using a Coulter counter and Coulter "Channelyzer." This was accomplished by fixing the cells with 40 µl of ice-cold isotonic phosphate buffered glutaraldehyde and refrigerating. The final concentration of the glutaraldehyde solution was adjusted with deionized water to give the same modal cell volume for both fixed and unfixed cells. This method was developed by Buckhold Shank et al. (198) specifically to measure osmotically induced changes in cell volume. The viability of cells used in this investigation were assayed by trypan blue dye exclusion using a modification of the technique developed by Gelsthorpe and Daughty (247). The sample was diluted to 1 x 10^5 - 5 x 10^6 cells/ml with saline. One hundred µl of this suspension was placed in a 0.5 ml microcentrifuge tube with 100 µl of 0.2% trypan blue. After five minutes at room temperature 200 µl of 1% BSA was added to adsorb any excess dye. After mixing, 100 µl of 10% acetic was added, the tube capped, and vortexed vigorously. The adsorption of the dye with BSA followed by the addition of acetic acid has two advantages over the conventional technique. The 2% acetic acid fixes the lymphocytes and lyses any erythrocytes in the suspension. Thus, one does not have to worry about making a correction for the RBCs and the cells do not need to be read immediately. If the latter

is an important consideration care should be taken to insure that the quantity of BSA added is sufficient to completely adsorb the free dye otherwise the viability values will change with time.

The functional integrity of lymphocytes following exposure to a pulsed electric field was monitored by measuring the ability of cells to undergo mitogen induced transformation to lymphoblasts using standard micro-culture techniques (248-252). Cells were exposed to the pulsed fields (and controls sham exposed) using the standard exposure cell which had been UV sterilized, rinsed with 70% ethanol, and fitted with a small plastic top to reduce the possibility of contamination during exposure (outside of the sterile hood). Following exposure, the sample was placed in a sterile 0.5 ml microcentrifuge tube. After all samples had been exposed (up to two hours) the cells were gently mixed using a vortex mixer and aliquots of the appropriate size were diluted in RPMI to give the necessary volume at a cell concentration of about 1.7×10^6 cells per ml. One hundred fifty µl of these lymphocyte suspensions was then dispensed (Titertek 12 channel pipettor) into the wells of a microtiter tray, each well already contained 50 µl of mitogen solution at the appropriate concentration

to give the desired final concentration. The microtiter trays were then placed in a controlled atmosphere incubator (37°C, 95% air, 5% CO_2, saturated humidity) for 72 hours. Either 24 hours (for initial experiments) or 4 hours before the end of the incubation, 50 µl of RPMI containing 1 µCi of ^{3}H-methyl-thymidine (2 or 10 Ci/mmole) was added to each well. At the end of the incubation period the cells were harvested using an automatic harvester capable of harvesting one (Otto Mfg. Co.) or two (Mash II, Microbiological Associates, Bethesda, MD) rows of 12 wells simultaneously. The medium and cells were suctioned out of each well through a piece of glass fiber filter paper then washed thoroughly (10x) with saline or deionized water (Tew, personal communication). The filter paper was dryed under heat lamps, the disc from each well was placed in a polyethylene "Bio-Vids" (Beckman Instruments), 3.5 ml of a Xylene based scintillation solution (4a20, Research Products International) was added and the vials counted in a Beckman model LSC-355 liquid scintillation counter.

Three mitogens were used in this study and each mitogen was used at three different concentrations plus zero mitogen to insure near optimal response. The mitogens and final concentrations used were Concanavalin

A (Con-A) (Sigma) used at 3 of the following concentrations: 20,10,5, 2.5, or 1.25 μg/ml; Phytohemagglutinin (PHA-P) (Difco) used at 3 of the following dilutions from stock: 1/100, 1/200, 1/400, 1/800; Lipopolysaccharide extracted from E. coli serotype 0111:B4 (LPS) (Difco No. 3122-25) used at 3 of the following concentrations: 800,400,200,100,50, or 25 μg/ml.

Each sample was assayed in quadruplicate so each 96 well plate held six groups, five exposed and one sham exposed control. The ^{3}H count rates were punched on paper tape by the scintillation counter, transferred to magnetic tape in the MCV data acquisition center, and sent to the VCU/VCCS Academic Computing Center where they were read into the IBM 370/168 and stored in disc file for processing. The data analysis for this study, including the randomization of exposure order and position on the microculture plates, was done with Statistical Analysis System (SAS), version 79.5.

The mitogenicity assays were analyzed using the following procedure. First, each microculture plate was scanned for outliers using the method of Bliss, Cochran, and Tukey (255). This method is based on the range, and was developed specifically to locate outliers in series of replicate samples. The importance of locating aberrant values when one is trying to obtain the best estimate of

an indicator of central tendency with small sample sizes has been discussed by Dixon (256). Frequently, the outliers located by following this procedure were located along the edges of the culture plate where one could also discard the values on methodological grounds. Following the elimination of outliers the analysis proceeded by locating the zero mitogen wells of the sham exposed control and taking their average. This average was defined as the background incorporation level for that microculture plate, and was subtracted from the cpm of each well on the plate, including the wells used to obtain the background value. The data from this type of mitogenicity assay have been shown to have a log-normal distribution (253), so the cpm-background value for each well was converted to its natural log. The quadruplicate values were then averaged and the exposed/control ratios were calculated for each electric field strength:mitogen concentration combination. The data were examined graphically and stored in a file on disc in the computer for further analysis at a later date. After several experiments had been run it was observed that there was a great experiment to experiment difference in the response levels, a problem that seems to be inherent in the assay (254). To assist in the evaluation of the results in light of this day to day variation the data

were pooled and subjected to a two way and a four way analysis of variance.

The functional integrity of Ehrlich ascites cells was assessed by injecting 10^6 exposed or control cells into the peritoneum of each of five mice. After five days the ascites was collected from each mouse, pooling the cells within each group. The pooled cells were then counted using a Coulter Counter (model ZH), and the results expressed as cells per mouse. In one experiment, 5 additional mice per treatment were injected with 10^6 cells. These mice were then allowed to live as long as possible and the mean survival time of each group was determined.

RESULTS[9]

Electric Field-Induced Potassium Efflux: Voltage Dependence

The exposure of mouse spleen lymphocytes to a single two microsecond high voltage electrical pulse resulted in an electric field dependent loss of intracellular potassium into the suspending medium, as indicated in Figure 3. The sigmoid shape of this curve is similar to that observed for the release of K^+ from erythrocytes and plant cells (3-6) and for the effect on the viability of microorganisms (1-4) exposed to pulsed electric fields. The field strength required to produce a 50% loss of intracellular potassium from mouse spleen lymphocytes in this study was 3.0 kV/cm, and the mean cell volume of these cells was 92 μm^3 corresponding to a cell radius of 2.79 μm. Substituting these values into equation XI, obtained by solving Laplace's equation for a dielectric sphere in a uniform electric field (257,258):

$$V_m = 1.5\ r\ E \qquad \text{IX}$$

where V_m is the transmembrane potential, r is the cell radius, and E is the electric field strength, gives a

---

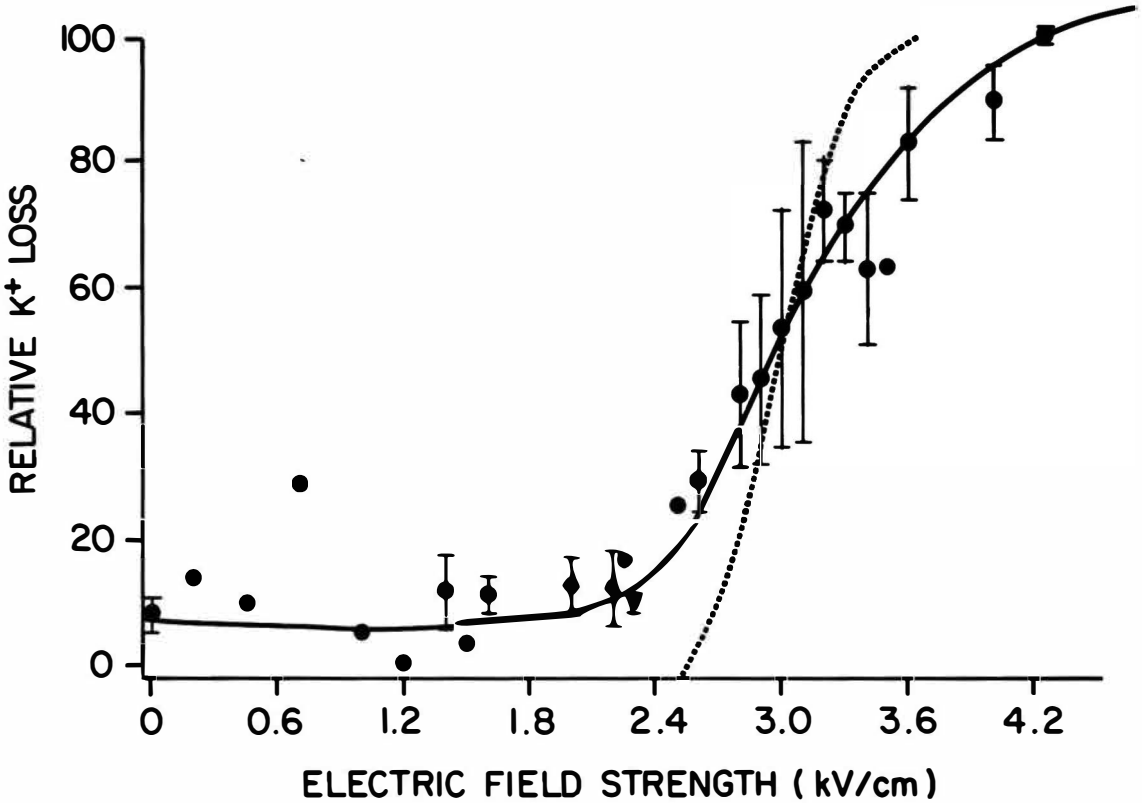
9. Unless otherwise indicated, the results are presented as the mean ± the standard error of values from 3-6 individual experiments. Values appearing as single points are values where replicates were not obtained. The lack of replicates was generally due to technical problems associated with the pulse amplifier.

value of 1.26 volts as the breakdown voltage for the plasma membrane of mouse spleen lymphocytes. This value is in general agreement with the breakdown voltage reported for other cell types (1-6). The dashed curve plotted in Figure 3 represents the K^+ release versus electric field strength curve that would be expected for a homogeneous population of cells with the same volume distribution of the mouse spleen cells used in these experiments. This curve was obtained by solving equation XI for E using the distribution of cell radii obtained from a typical sample, as measured with a Coulter Counter, and the value of 1.26 volts obtained for V_m, above. These results were then used to plot the percent of cells with an induced transmembrane potential greater than or equal to 1.26 volts as a function of the electric field strength. Although the agreement between this theoretical curve and the experimental data is not quantitative, it does indicate that the size distribution of the cells in the exposed sample was a probable cause of the shape of the experimental curve.

Electric Field-Induced Potassium Efflux: Pulse Duration Dependence

Figure 4 shows the results from experiments in which mouse spleen cell suspensions were exposed to a single 1.5 kV (3.0 kV/cm) pulses of various pulse

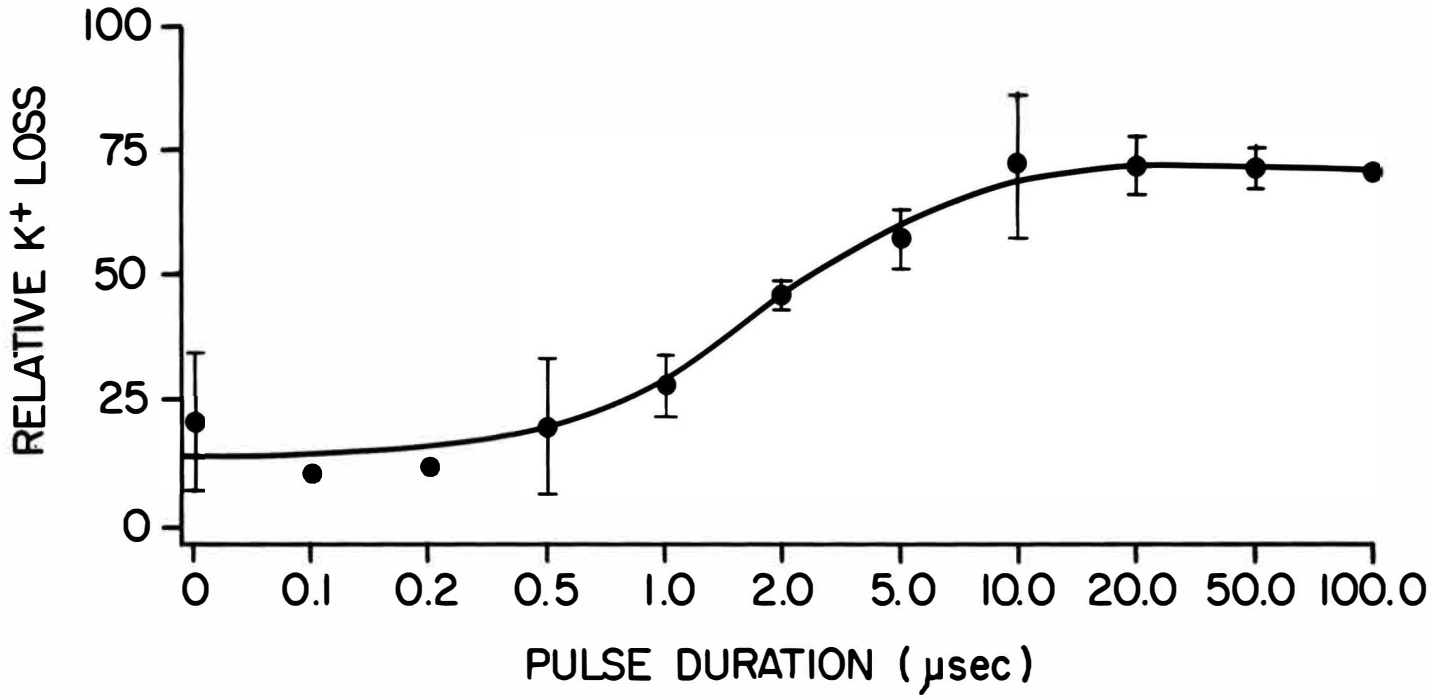
Figure 3: The relative loss of intracellular potassium into the exposure medium produced by a single 2 μ pulse, plotted as a function of the electric field strength of the pulse. The dashed curve represents the curve that would be expected if the entire distribution of the potassium loss was due to the size distribution of the cells in the sample.



Relative potassium loss from cells as a function of electric field strength.

Figure 3

Figure 4: The relative potassium loss produced by a single 3.0 kV/cm pulse, plotted as a function of the pulse duration.



Relative potassium loss from mouse spleen cells as a function of pulse duration, in microseconds.

Figure 4

durations. The data have been plotted on a quasi-log time scale in order to include 0 μsec (i.e. sham exposed cells) and the full range of pulse durations used, 0.1-100 μsec. There was little or no potassium release from cells exposed to pulses with durations less than 0.5 μsec; between 0.5 and 1.0 sec there was a significant increase in the percent of intracellular potassium loss which reached a plateau between 5.0 and 10.0 μsec. This plateau extends at least to 100 μsec.[10] The dramatic nature of the pulse duration dependence of field-induced cellular damage is also indicated by Coulter volume spectroscopy of cells from the above samples that were fixed with isotonic gluteraldehyde approximately one hour after exposure to the pulsed field (Fig. 5). These curves represent the relative size distribution of the mouse spleen lymphocytes in a given sample. The curves were made by counting each sample until the number of cells in the modal channel equaled 1,000; consequently, they do not necessarily reflect the absolute number of cells in any given portion of the volume spectrum.

There are several important points to note in these volume spectra. The first thing to notice in the various

---

10. With the exposure system used in this study Joule heating of the bulk sample becomes a problem for pulse widths longer than 100 μsec.

Figure 5: Coulter volume spectra of cells fixed in isotonic glutaraldehyde following exposure to a single 3.0 kV/cm electrical pulse of various pulse durations.

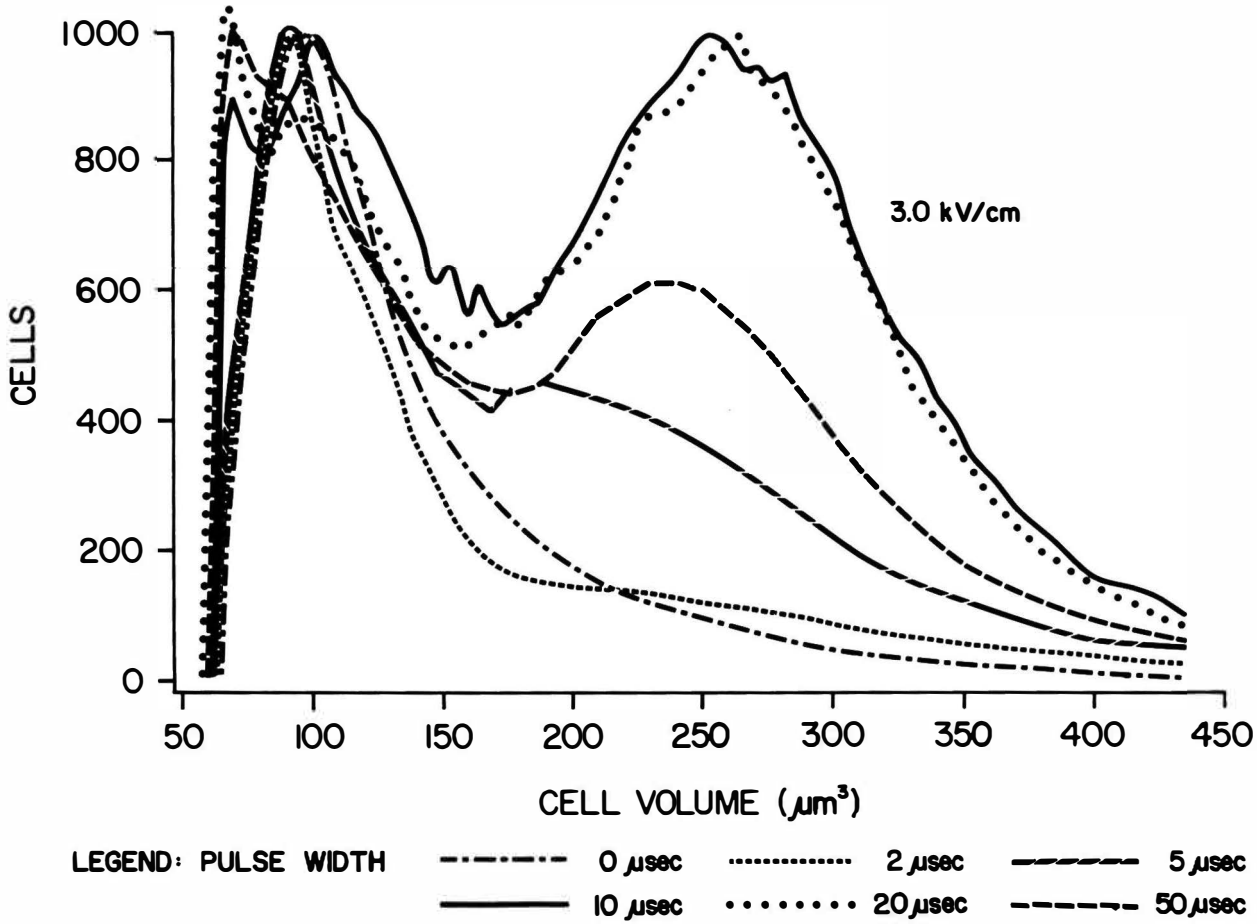

1000
800
600
400
200
0
CELLS
3.0 kV/cm
50
100
150
200
250
300
350
400
450
CELL VOLUME (μm³)
LEGEND: PULSE WIDTH
0 μsec
2 μsec
5 μsec
10 μsec
20 μsec
50 μsec

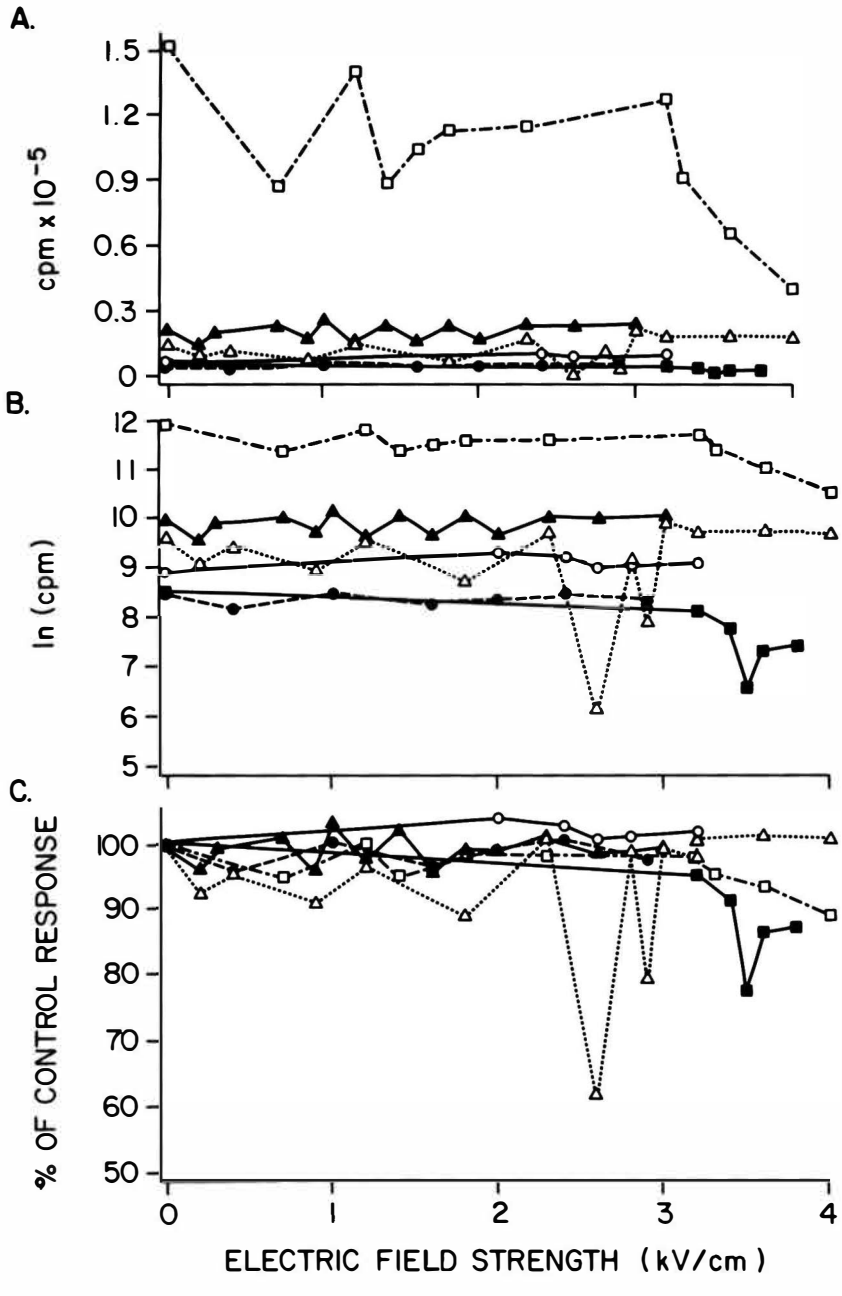
Figure 5

size distributions is the appearance of a second peak in the size distribution curves of cells exposed to pulse of 5 μsec. or longer. This peak indicates the presence of a large number of cells which have swollen after exposure to the 3 kV/cm electrical pulse and were unable to attain normal volume control even after one hour at room temperature. The second change in the volume distribution following exposure to a pulsed electric field was the increase in the number of small particles in the samples exposed to long pulse lengths (i.e. $\geq$ 5 μsec), due to the increased amount of cell debris in these samples. This increase in cell debris was seen to some extent in all of the exposed samples, but it is best illustrated by the 50 μsec sample where the decrease in the proportion of swollen cells compared with the 10 and 20 μsec samples was accompanied by a large increase in the size of the debris peak. Another point to be noted, particularly in the shorter pulse length samples is that the increases in the debris and swollen cell peaks were reflected by concomitant decreases in the size of the large volume area of the normal cell peak. It is also of interest to note that the volume spectra appear to provide a more graded indication of the extent of cell injury in cells exposed to pulse widths between 0.5 and 50 μsec than the potassium release data.

Effect of Pulsed Electric Fields on Mitogenicity

Studies in which the effects of exposure to pulsed electric fields on mitogenicity of mouse spleen lymphocytes was assayed by measuring the amount of tritiated thymidine incorporated into the cells during the last 4 or 12 hours of a 72 hour incubation in the presence of either Con-A or PHA-P (T-cell mitogens) or LPS (B-cell mitogen) were undertaken to obtain information regarding the functional integrity of the cells following such an exposure. The results from these experiments were very erratic with considerable experiment to experiment variation. This is illustrated in Figures 6-8 which represent typical data for each mitogen with each symbol representing results from a different experiment. In these scatter plots (Figs. 6-8a), the cpm incorporated into cells incubated with the mitogen after exposure to a 2 μsec pulse is presented as a function of the electric field strength. The variability of this data is greatly reduced by transforming the data from cpm to the natural log of the cpm (Figs. 6-8b). In Figures 6-8c the ratio of the responses exposed to sham exposed [$\ln(cpm)_e/\ln(cpm)_c$] is plotted as a function of field strength. As indicated in these figures, the results from several experiments suggest a modest enhancement of mitogenicity at the lower field strengths and a decreased response to

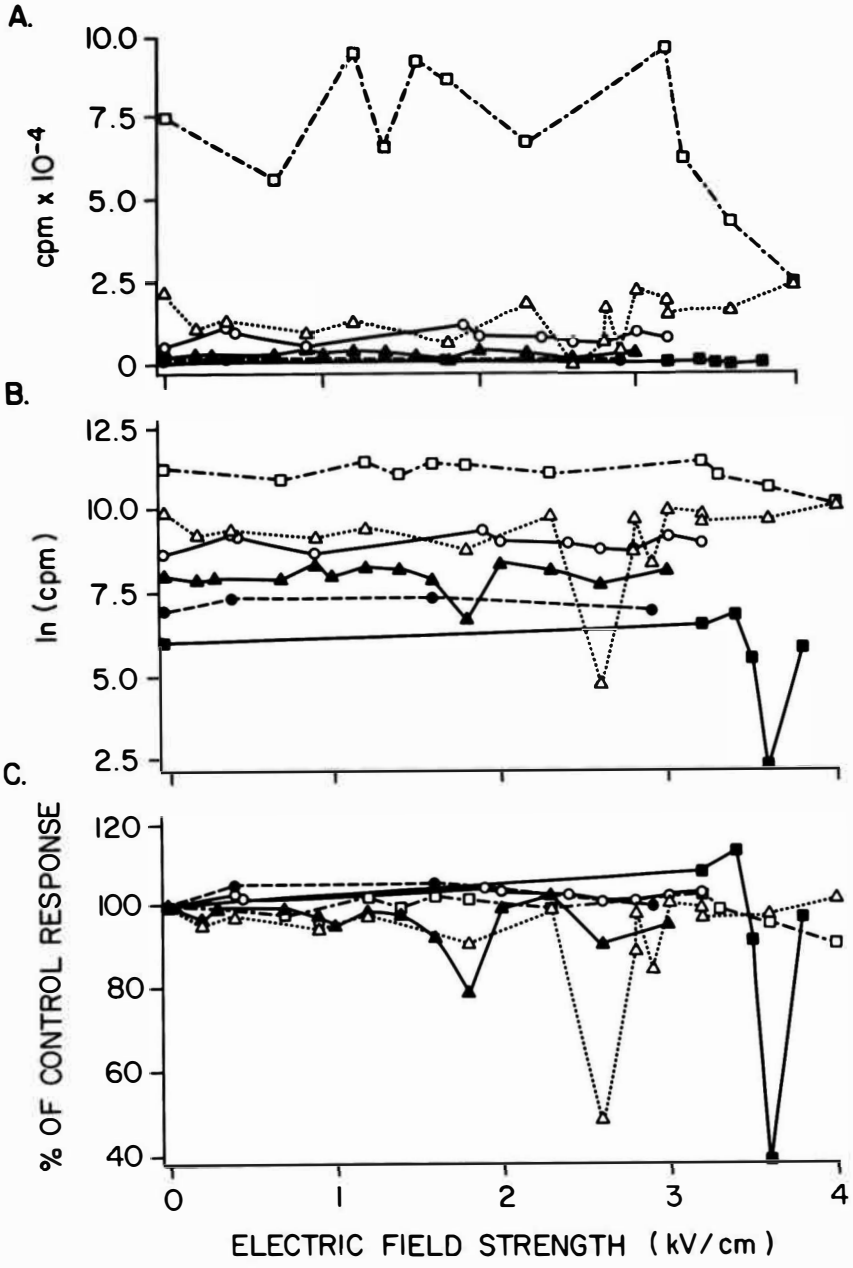
Figure 6: A scatter plot of the response of mouse spleen lymphocytes cultured in the presence of 5.0 μgm/ml Con-A following exposure to a single 2 μsec pulse plotted as a function of electric field strength. a) cpm, b) ln (cpm); c) ratio of exposed response to control response as percent. Each symbol represents a different experiment.



2 μsec data--Con A

Figure 6

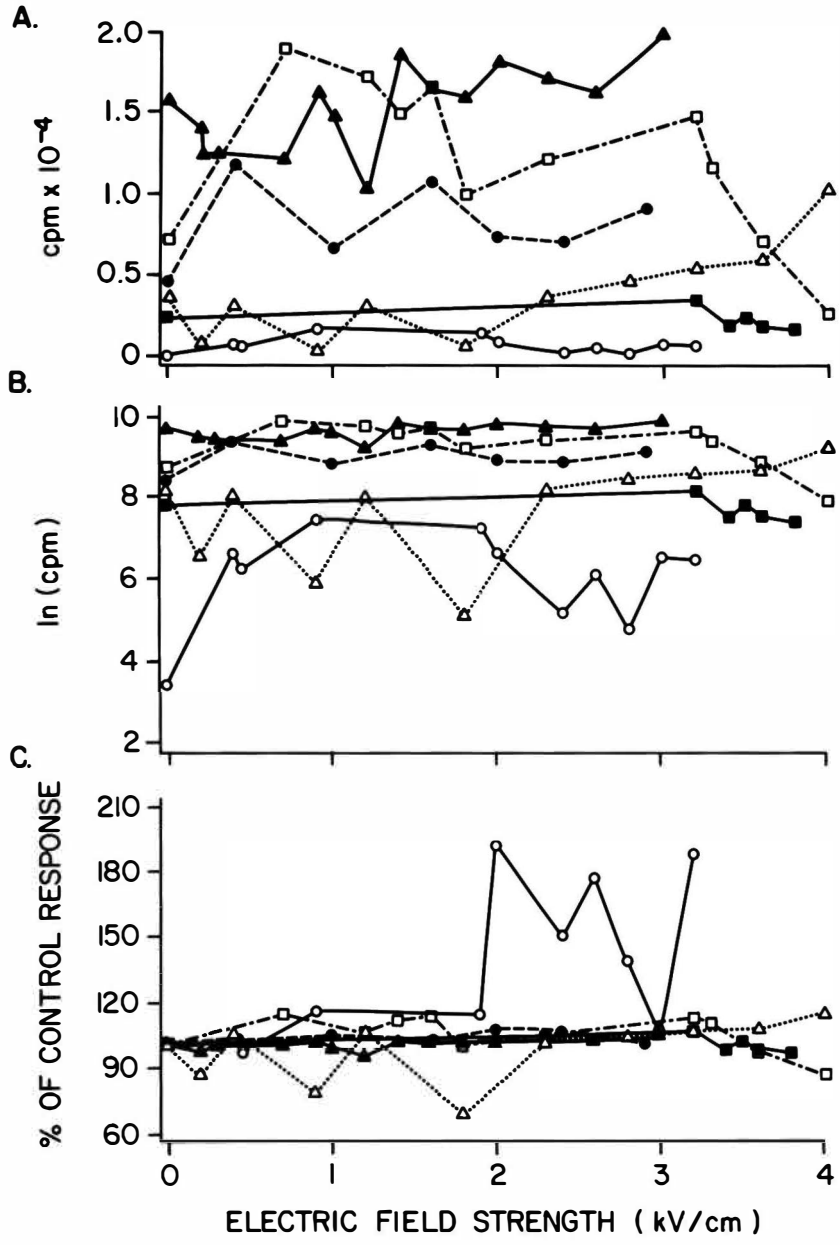
Figure 7: A scatter plot of the response of mouse spleen lymphocytes cultured in the presence of a 1/200 dilution of PHA-P following exposure to a single 2 μsec pulse plotted as a function of electric field strength. a) cpm, b) ln (cpm); c) ratio of exposed response to control response as percent. Each symbol represents a different experiment.



2 μsec data--PHA-P

Figure 7

Figure 8: A scatter plot of the response of mouse spleen lymphocytes cultured in the presence of 400 μgm/ml LPS following exposure to a single 2 μsec pulse plotted as a function of electric field strength. a) cpm, b) ln (cpm); c) ratio of exposed response to control response as percent. Each symbol represents a different experiment.



2 μsec -- LPS

Figure 8

mitogenic stimulation at higher voltages. These effects appear to be more pronounced in those cells cultured in the presence of LPS, so a more detailed analysis of this data is presented here; although, the conclusions regarding the LPS-stimulated cells also hold true for the cells cultured in the presence of Con-A or PHA-P following exposure to two microsecond electrical pulses.

Mitogenicity Data Transformation

Figures 9-12 represent the data from each of the concentrations of LPS used in these experiements (i.e. 0, 100, 200, 400 μgm/ml). The data are plotted as the mean value plus or minus the standard error of the mean. Comparing the untransformed data (cpm vs kV/cm) (Figs. 9-12a) with the transformed data (ln(cpm) vs kV/cm) (Figs. 9-12b) clearly illustrates the improvement of the error structure of the data obtained by using the natural logarithmic transformation. This reduction in the variability of the transformed data is related to the fact that mitogenicity assay data are log-normally distributed, as previously reported in the literature (252). This requires the log-transformations of the data to obtain valid inferences using the standard parametric statistical tests.

Figure 9: Summary of data from mouse spleen cells cultured in the presence of 0 μgm/ml LPS following exposure to a single 2 μsec electrical impulse. The response is plotted as a function of electric field strength, each point is the mean of 2-6 experiments, error bars are standard errors. a) cpm; b) ln (cpm); c) ratio of exposed response to control response $[\ln(\text{cpm})_e / \ln(\text{cpm})_c]$ as percent.

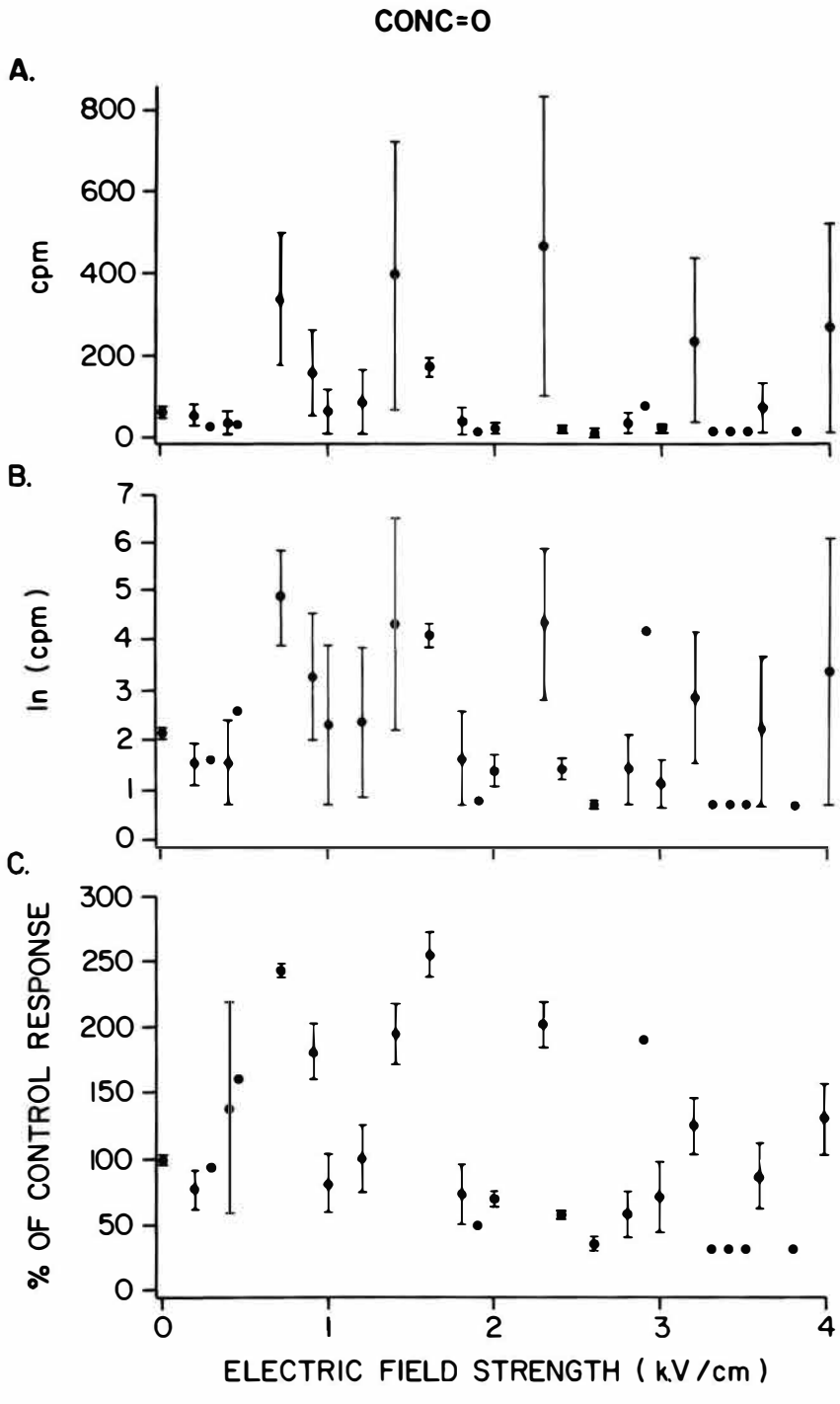

CONC=0
A.
800
600
400
200
0
cpm
B.
7
6
5
4
3
2
1
0
ln (cpm)
C.
300
250
200
150
100
50
0
% OF CONTROL RESPONSE
0
1
2
3
4
ELECTRIC FIELD STRENGTH (kV/cm)
2 μsec data--LPS

Figure 9

Figure 10: Summary of data from mouse spleen cells cultured in the presence of 100 µgm/ml LPS following exposure to a single 2 µsec electrical impulse. The response is plotted as a function of electric field strength, each point is the mean of 2-6 experiments, error bars are standard errors. a) cpm; b) ln (cpm); c) ratio of exposed response to control response as percent.

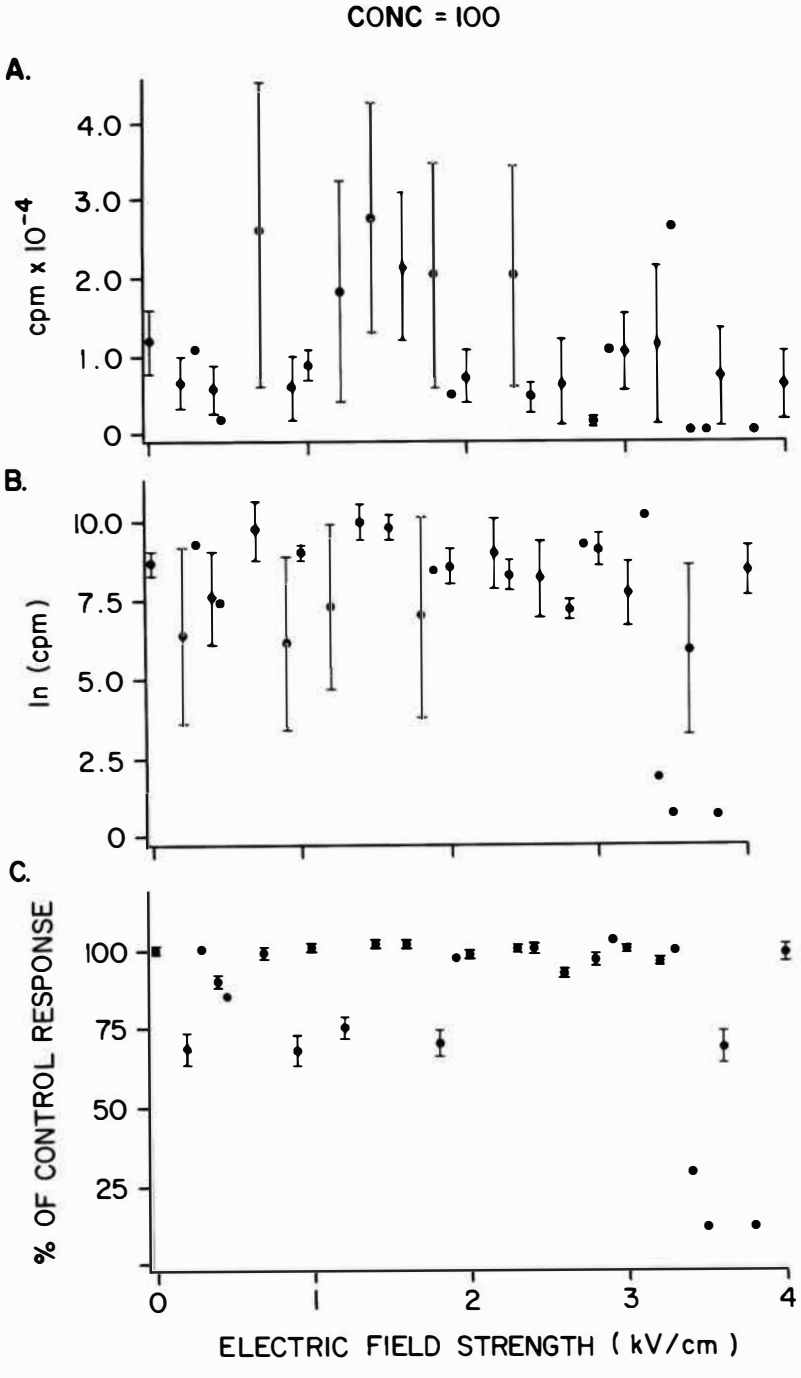

CONC = 100
A.
cpm x 10^{-4}
4.0
3.0
2.0
1.0
0
B.
ln (cpm)
10.0
7.5
5.0
2.5
0
C.
% OF CONTROL RESPONSE
100
75
50
25
0
1
2
3
4
ELECTRIC FIELD STRENGTH (kV/cm)
2 μsec data--LPS

Figure 10

Figure 11: Summary of data from mouse spleen cells cultured in the presence of 200 μgm/ml LPS following exposure to a single 2 μsec electrical impulse. The response is plotted as a function of electric field strength, each point is the mean of 2-6 experiments, error bars are standard errors. a) cpm; b) ln (cpm); c) ratio of exposed response to control response as percent.

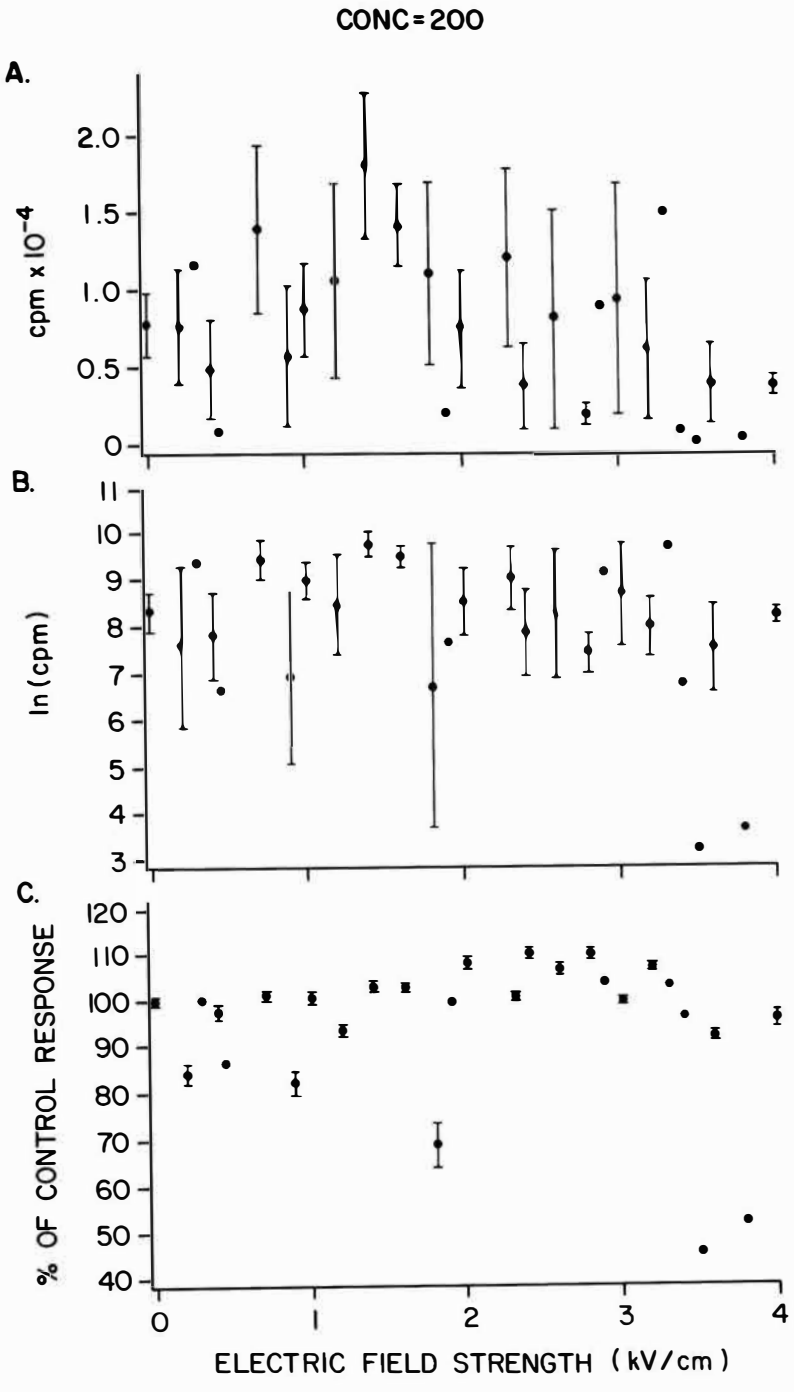
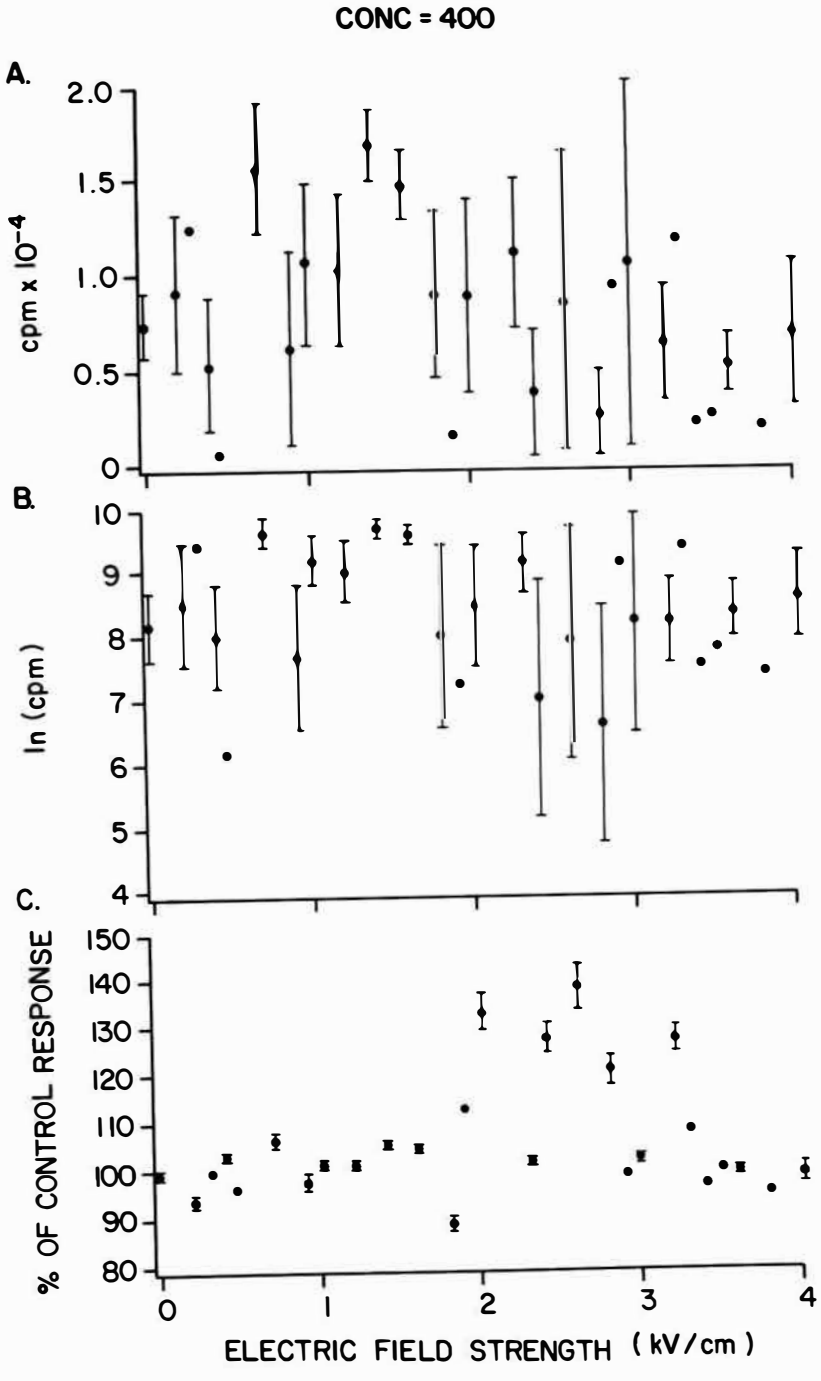

CONC=200
A.
cpm x 10⁻⁴
2.0
1.5
1.0
0.5
0
B.
ln(cpm)
11
10
9
8
7
6
5
4
3
C.
% OF CONTROL RESPONSE
120
110
100
90
80
70
60
50
40
0
1
2
3
4
ELECTRIC FIELD STRENGTH (kV/cm)
2 μsec data--LPS

Figure 11

Figure 12: Summary of data from mouse spleen cells cultured in the presence of 400 μgm/ml LPS following exposure to a single 2 μsec electrical impulse. The response is plotted as a function of electric field strength, each point is the mean of 2-6 experiments, error bars are standard errors. a) cpm; b) ln (cpm); c) ratio of exposed response to control response as percent.



2 μsec data--LPS

Figure 12

Statistical Analysis

A two-factor analysis of variance of the log-transformed data indicated that the experiment-to-experiment variation was a significant factor at all four concentrations of mitogen ($p<0.05$), and the electric field was not a statistically significant factor in any of these sets of data. Similar results were obtained from the analysis of variance of both the Con-A and PHA-P data.

Effects of Longer Pulse Duration on Mitogenic Response

Considering the dramatic difference in the effectiveness of 2 and 10 μsec pulse widths on the release of intracellular potassium (Fig. 4), a series of experiments was conducted to determine if exposure of mouse spleen cells to 10 μsec pulses prior to incubation would yield more definitive data. These experiments were restricted to LPS stimulation in order to reduce the technical complexity of the experiments. The results of these experiments are summarized in Figures 13-16. In these experiments samples from each exposure level were fixed with gluteraldehyde and subsequently counted with a Coulter counter. The cell concentration data were then used to normalize the data to account for differences in the cell concentration of the individual samples. Normalization was accomplished by dividing the cpm by

the appropriate cell concentration to obtain cpm per 10^5 cells at the beginning of the incubation, and then taking the natural log transform of these data. Figures 13-16a show the ln (cpm/10^5 cells) as a function of field strength, and Figures 13-16b present the same data using the ratio of exposed cells to control cells. A marked decrease in the response observed at field strengths greater than 2.4 kV/cm is seen in these data. Analysis of variance similar to that employed with the two microsecond data indicated that the experiment-to-experiment term also had a significant effect on these data; however, both the data at LPS concentrations of 200 μgm/ml and 800 μgm/ml have a statistically significant electric field effect ($p<0.0001$); at a LPS concentration of 400 μgm/ml the significance of the electric field effect is reduced to marginal levels ($p=0.1002$). These experiments are interpreted as indicating a significant degree of cell killing at field strengths above 2.4 kV/cm.

As the purpose of testing the mitogenicity of the mouse spleen cells was to assess the effect of exposure to a pulsed electric field on the physiological integrity of these cells, it is instructive to examine the ratio of the mitogenic response of the exposed cells to the

Figure 13: Summary of data from mouse spleen cells cultured in the presence of 0 μg/ml LPS following exposure to a single 10 μsec electrical impulse. The response is plotted as a function of electric field strength, each point is the mean of 2-6 experiments, error bars are standard errors. a) $\ln(\text{cpm}/10^5 \text{ cells})$; b) ratio of exposed response to control response as percent.

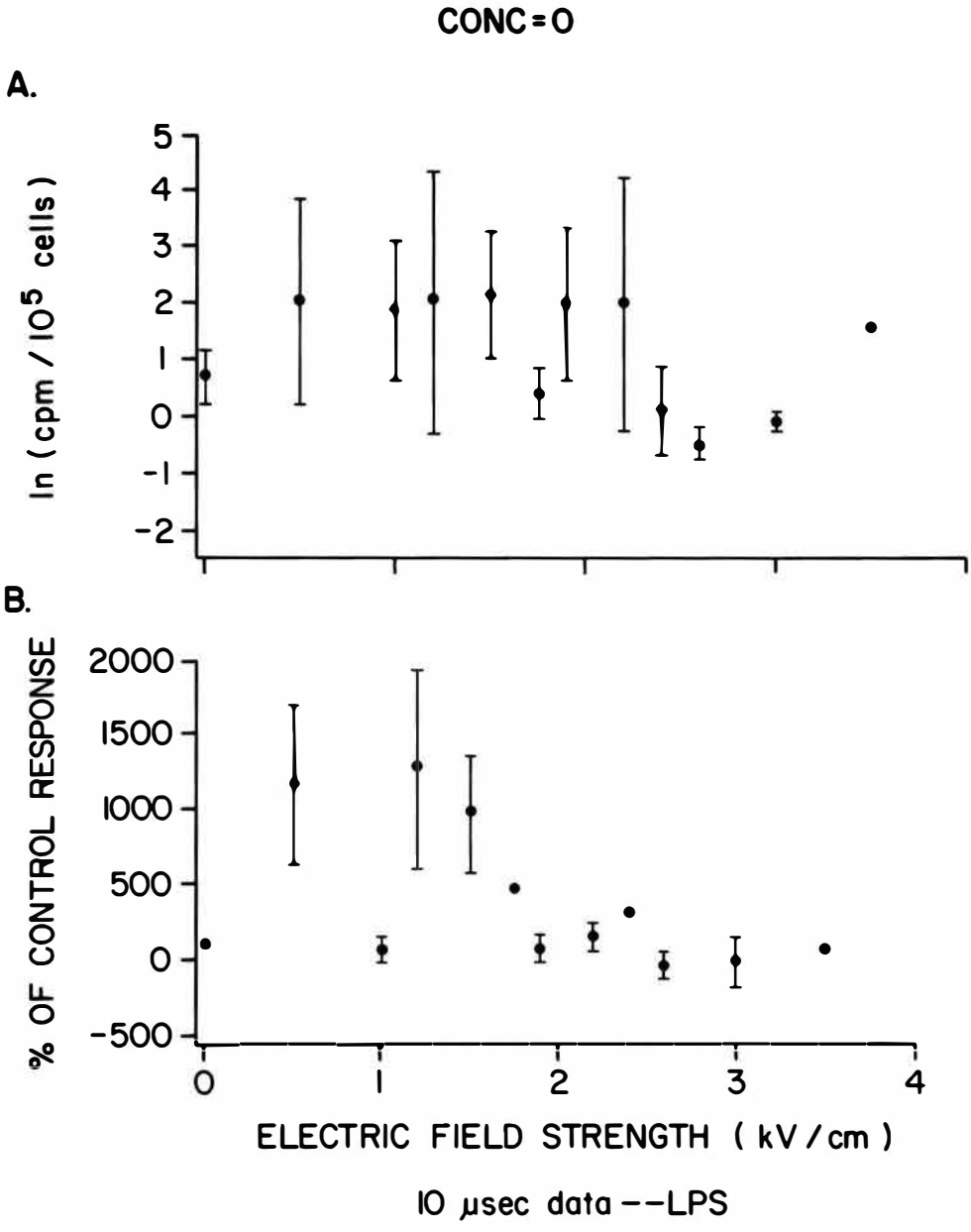

CONC=0
A.
ln (cpm / 10^5 cells)
5
4
3
2
1
0
-1
-2
B.
% OF CONTROL RESPONSE
2000
1500
1000
500
0
-500
0
1
2
3
4
ELECTRIC FIELD STRENGTH (kV / cm)
10 μsec data --LPS

Figure 13

Figure 14: Summary of data from mouse spleen cells cultured in the presence of 200 μg/ml LPS following exposure to a single 10 μsec electrical impulse. The response is plotted as a function of electric field strength, each point is the mean of 2-6 experiments, error bars are standard errors. a) ln(cpm/10^5 cells); b) ratio of exposed response to control response as percent.

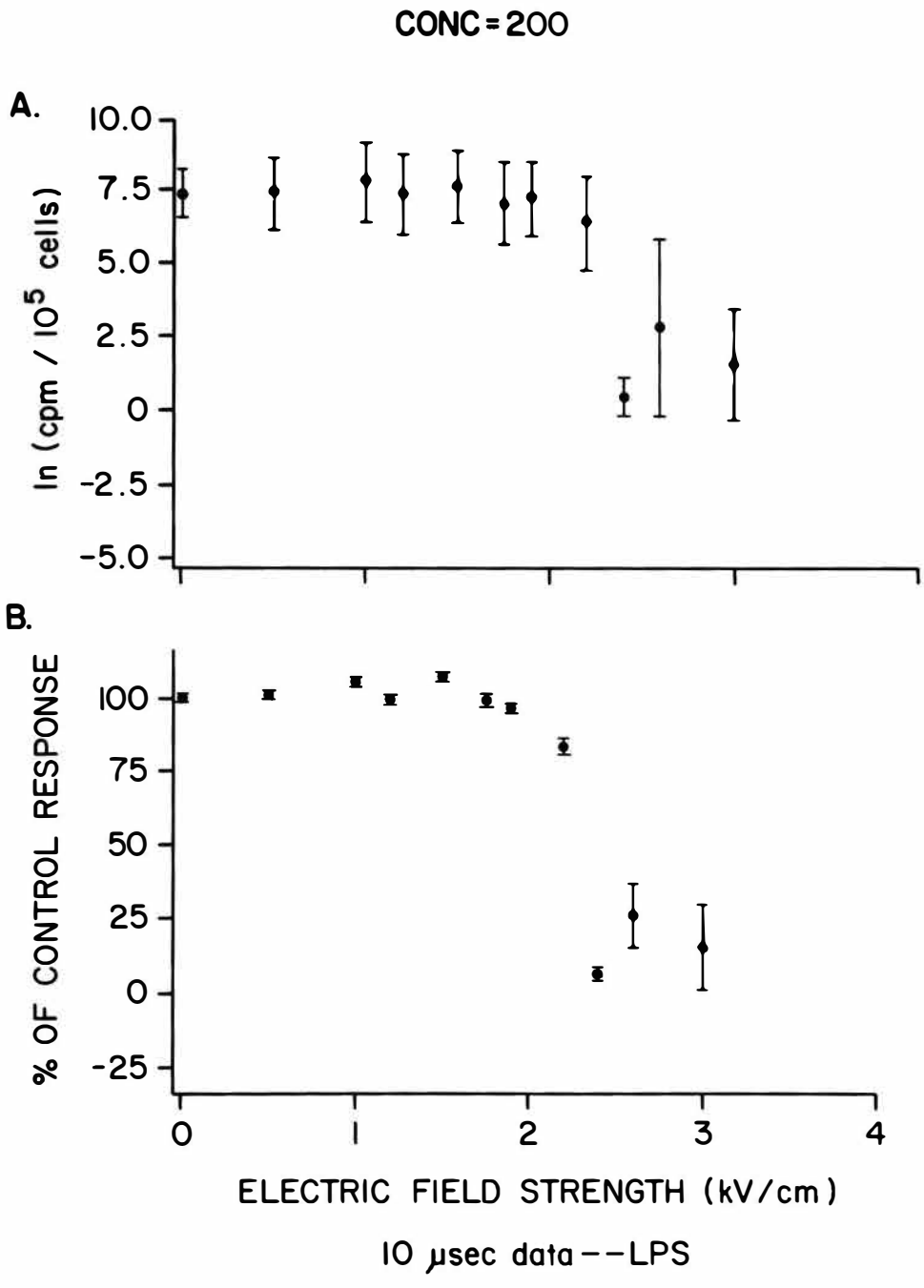

CONC = 200
A.
10.0
7.5
5.0
2.5
0
-2.5
-5.0
ln (cpm / 10^5 cells)
B.
100
75
50
25
0
-25
% OF CONTROL RESPONSE
0
1
2
3
4
ELECTRIC FIELD STRENGTH (kV/cm)
10 μsec data--LPS

Figure 14

Figure 15: Summary of data from mouse spleen cells cultured in the presence of 400 μg/ml LPS following exposure to a single 10 μsec electrical impulse. The response is plotted as a function of electric field strength, each point is the mean of 2-6 experiments, error bars are standard errors. a) $\ln(cpm/10^5)$; b) ratio of exposed response to control response as percent.

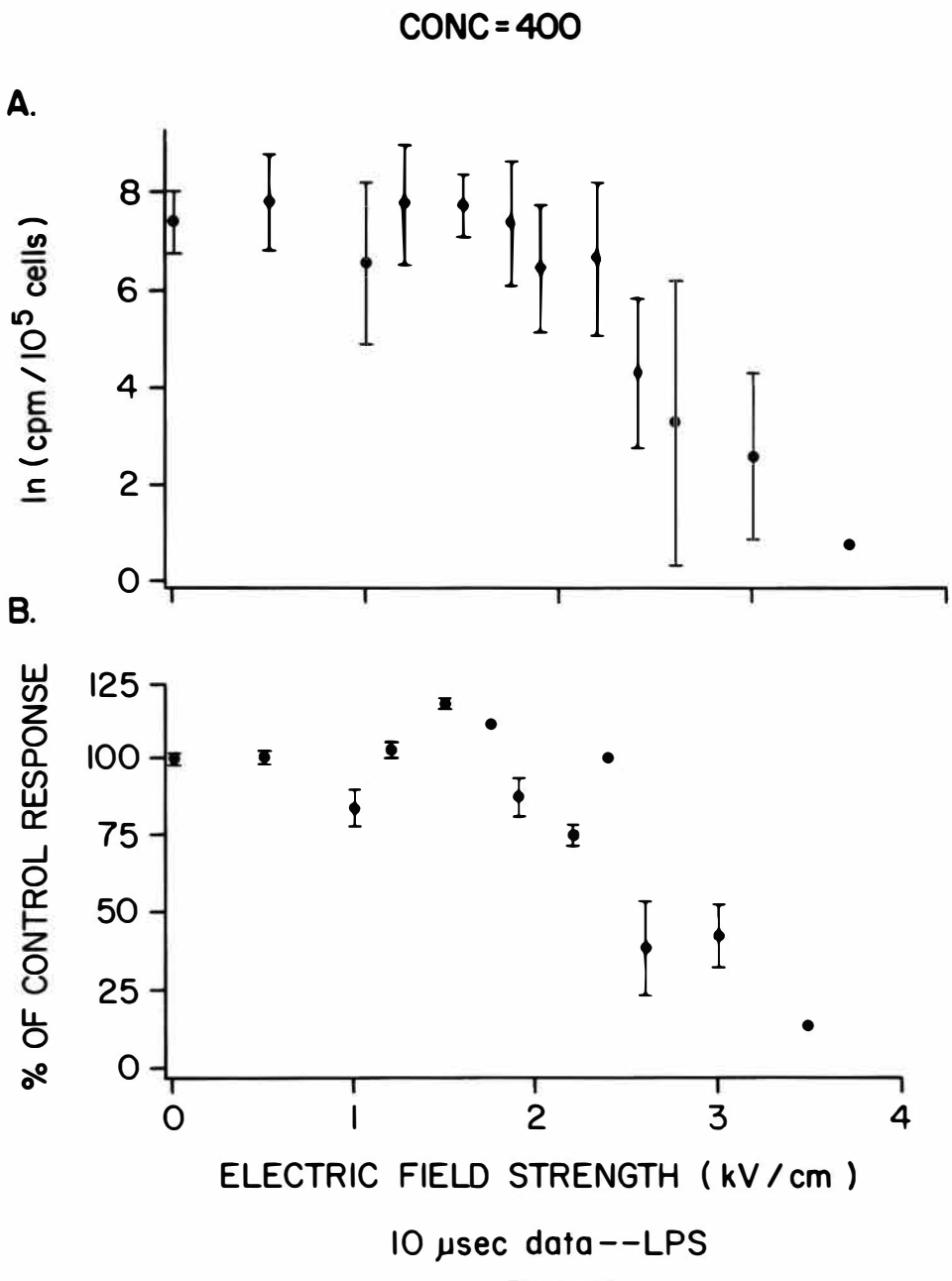

CONC = 400
A.
ln (cpm / 10^5 cells)
0
2
4
6
8
B.
% OF CONTROL RESPONSE
0
25
50
75
100
125
0
1
2
3
4
ELECTRIC FIELD STRENGTH (kV / cm)
10 μsec data--LPS

Figure 15

Figure 16: Summary of data from mouse spleen cells cultured in the presence of 800 μg/ml LPS following exposure to a single 10 μsec electrical impulse. The response is plotted as a function of electric field strength, each point is the mean of 2-6 experiments, error bars are standard errors. a) $\ln(cpm/10^5)$; b) ratio of exposed response to control response as percent.

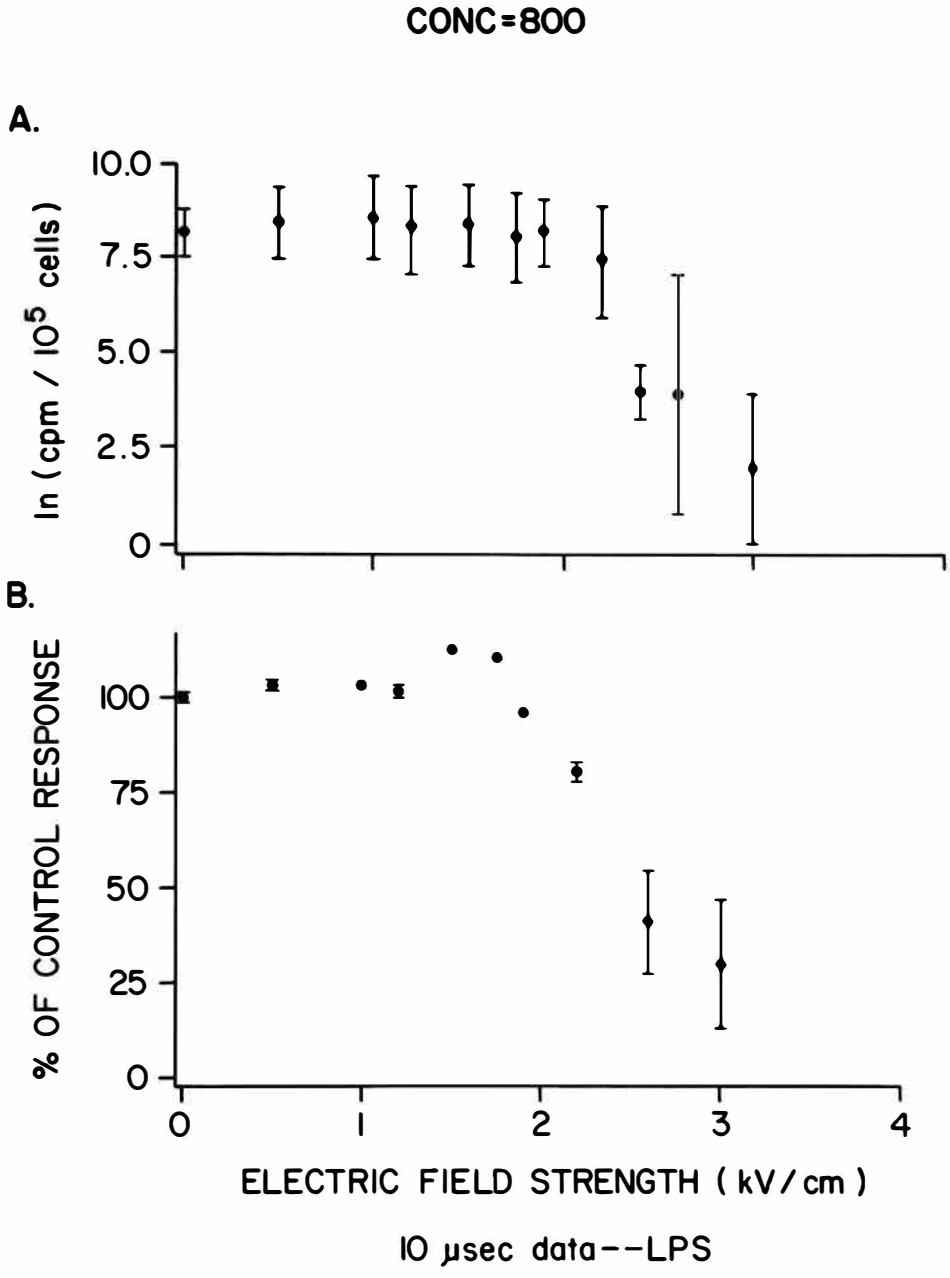

CONC=800
A.
10.0
7.5
5.0
2.5
0
ln (cpm / 10^5 cells)
B.
100
75
50
25
0
% OF CONTROL RESPONSE
0
1
2
3
4
ELECTRIC FIELD STRENGTH (kV/cm)
10 µsec data--LPS

Figure 16

response observed for the sham exposed controls. The use of ratios requires caution and the interpretation of such data must include consideration of the absolute level of the response. Taking both factors into consideration reveals that there is a persistent enhancement of cell growth in those cases where the response level is relatively low (i.e. <1000 cpm). This includes cells cultured without any mitogen and cells cultured for long periods of time (Fig. 17). The lack of statistical significance which is related to the low level of tritium incorporation makes it impossible to draw any definitive conclusions from these data, but the persistence of the effect and results reported by others regarding electrically stimulated growth make these interesting observations that will be considered in greater detail in the discussion.

Effects of Pulsed Fields on Lymphocyte Viability

Lymphocyte viability at 2 hours post-exposure to a two microsecond voltage pulse and on replicate samples incubated at 37°C in tissue culture medium for an additional two hours were determined using the vital stain trypan blue. The samples kept at room temperature for two hours showed a small field dependent decrease in viability (90-64%), while no differences in the viability (80-84%) of those cells incubated at 37°C for an

Figure 17: The response of mouse spleen lymphocytes cultured in 400 μgm/ml LPS plotted as a function of hours in culture.

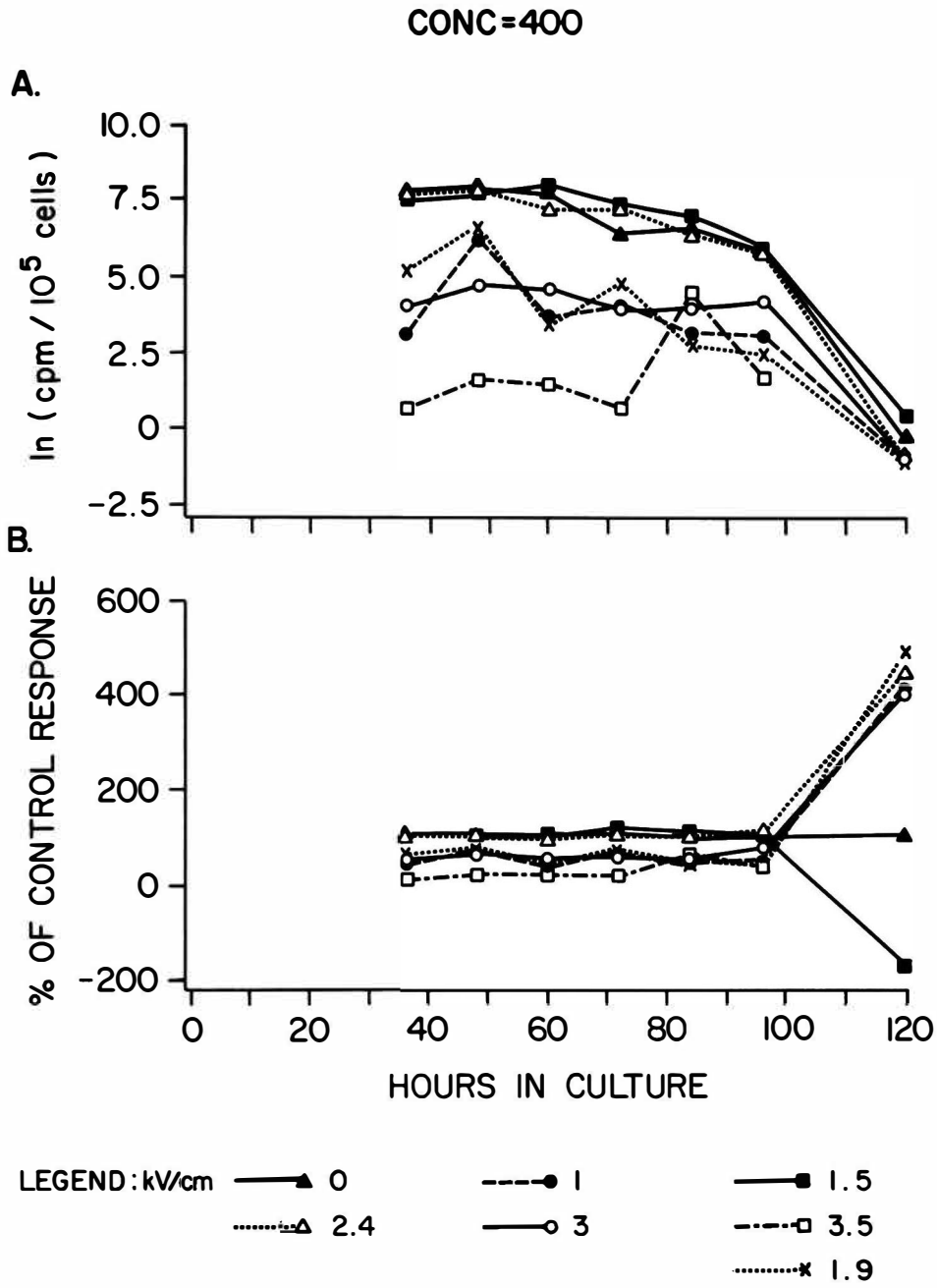

CONC=400
A.
10.0
7.5
5.0
2.5
0
-2.5
ln (cpm / 10^5 cells)
B.
600
400
200
0
-200
% OF CONTROL RESPONSE
0
20
40
60
80
100
120
HOURS IN CULTURE
LEGEND: kV/cm
0
1
1.5
2.4
3
3.5
1.9
10 µsec data--LPS

Figure 17

additional two hours were observed. These results are in good agreement with the observations of Mild et al. (259) who studied trypan blue uptake by human leukocytes exposed to electrical pulses with amplitudes of up to 30 kV/cm. This data also suggests that cellular damage from two microsecond pulses are reversible when the cells are incubated at 37°C. Mild and his colleagues also found no differences in the effects of 1 or 10 pulses at field strengths of less than 5 kV/cm which agrees with observations made on the mitogenicity of mouse spleen cells exposed to 0,1,2,5,10, or 20 pulses at 1 pulse per second.[11]

Effects of Pulsed Electric Field on Ehrlich Ascites Tumor Cells

A series of preliminary experiments was done to assess the effect of 10 microsecond high voltage pulses on Ehrlich ascites tumor cells. No significant differences were observed between the tumorigenicity of cells exposed to pulsed electric fields and sham exposed cells. This was true for both the number of cells harvested in the ascites fluid five days after the

---

11. Data not included as there were no differences in the response levels observed for these samples.

injection of mice with the treated cells (10^6 cells/ mouse, i.p.) and for the mean survival time of mice receiving similar injections (Fig. 18). In a parallel experiment the potassium release from Ehrlich ascites cells was measured as a function of electric field strength. These data presented in Figure 19 are of interest due to the relatively large size of Ehrlich ascites cells, 2,016 μm^3 corresponding to a modal radius of 6.32 μm. The unusual nature of these observations is seen by substituting the field strength required for a 50% release of intracellular K^+ (2.5 kV/cm) and this mean cell radius into equation IX and solving for V_m, the critical breakdown voltage of the cell membrane. This calculation gives a value of 2.37 volts compared to the frequently cited value of about 1 volt as the membrane breakdown voltage for most cells (4).

Figure 18: Bar chart of the number of Ehrlich ascites cells harvested 5 days after injection of 10^6 cells per mouse. The numbers from peritoneal ascites fluid in parenthesis above each bar represents the mean survival time in days of 5 additional mice injected with 10^6 cells/mouse from the same samples in those experiments were this determination was made.

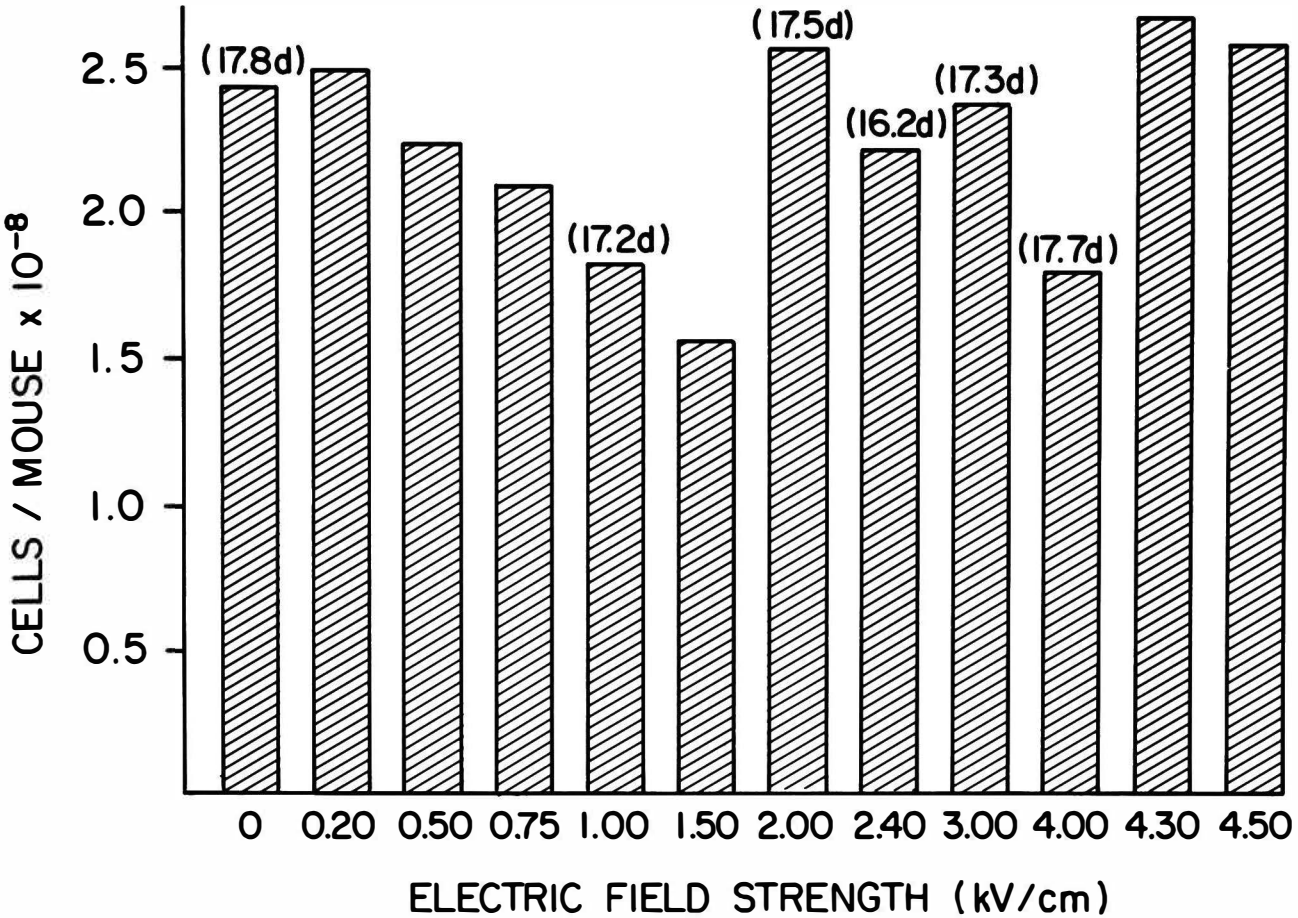
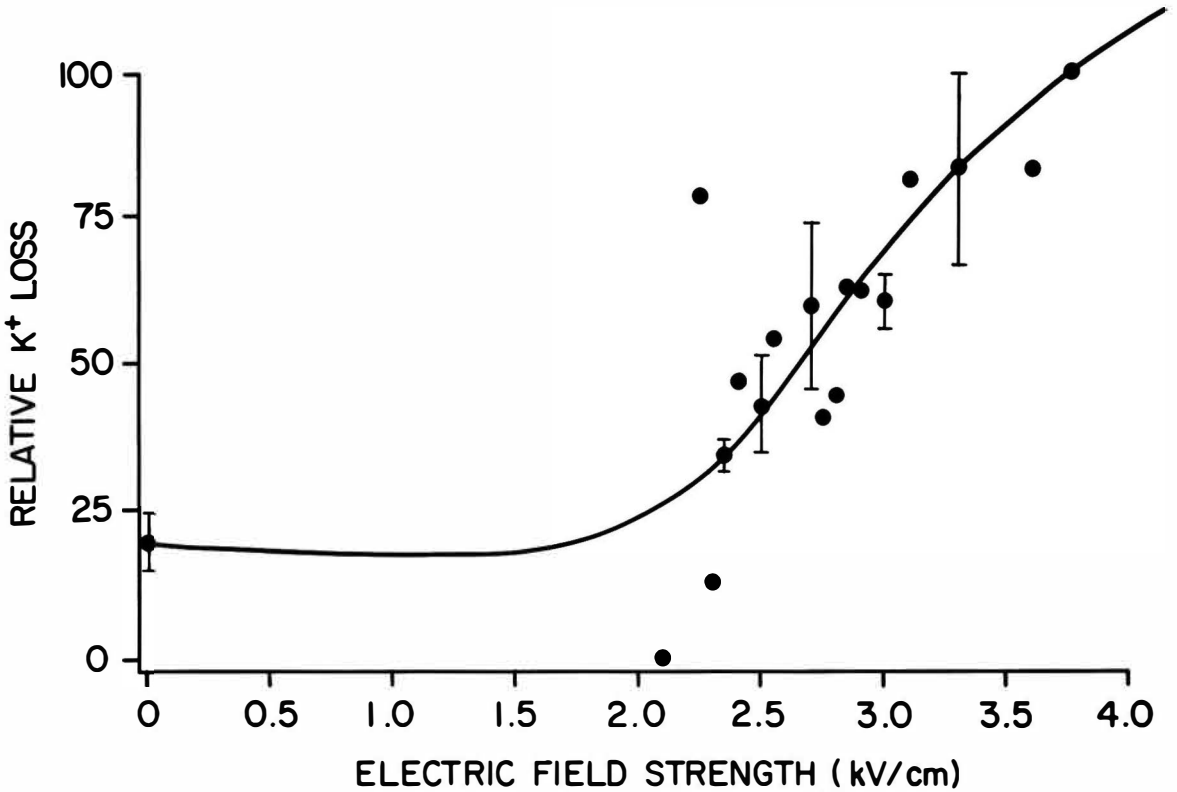

(17.8d)
(17.5d)
(17.3d)
(16.2d)
(17.2d)
(17.7d)
2.5
2.0
1.5
1.0
0.5
CELLS / MOUSE x 10⁻⁸
0
0.20
0.50
0.75
1.00
1.50
2.00
2.40
3.00
4.00
4.30
4.50
ELECTRIC FIELD STRENGTH (kV/cm)

Figure 18

Figure 19: The relative percent of intracellular potassium released into the medium by Ehrlich ascites cells following exposure to a single 10 μsec pulse at the indicated field strength.



Relative potassium loss from cells as a function of electric field strength.

Figure 19

DISCUSSION

On the basis of the experimental results reported here, the only inference that can be made with any degree of confidence is that mouse spleen lymphocytes respond to a high voltage electrical pulse in a manner that is both qualitatively and quantitatively similar to the response of mammalian erythrocytes and other eukaryotic cells (1-6). This response is characterized by a sigmoidal, field strength dependent cell injury curve, as indicated by the loss of intracellular potassium into the exposure medium. The loss of intracellular potassium (i.e. cell injury) also exhibits a strong dependence on the pulse duration between 0.5 and 10 μsec. This interpretation of the potassium release data is supported by the response of mouse spleen lymphocytes to the stimulation of these cells by the B-cell mitogen LPS following exposure to a single 10 μsec electric impulse. In this case there is a significant field strength dependent decrease in the amount of tritiated thymidine incorporated into cells following exposure to a 10 μsec electrical pulse at field strengths greater than 2.4 kV/cm. This observation is, in some cases, supported by the results of the mitogenic assays done with mouse spleen cells following exposure to a single 2 μsec pulse. Unfortunately, these results are subject to variation such that no statistically significant effect is seen when the data are pooled. This high

degree of variability undoubtedly results from the combined influence of factors related to both the technique itself, and to the experimental protocol used in this investigation.

A high degree of variability seems to be inherent in mitogenic assays in general (253,254), and was a factor that was not fully appreciated in the planning of this study. A related variable that was not controlled for in this study was the variation in the cell-type distribution from experiment to experiment and from sample to sample. The number of cells of each type (e.g. B and T lymphocytes, monocytes, polymorphonuclear leukocytes, and erythrocytes) obtained in any given preparation will be a function of the age, sex, immune status, strain, number, etc. of the mice used. Differences in the T:B:monocyte ratio from sample to sample are likely to have been a factor in both the intra- and the inter-experiment variation. Such variations have previously been described (254). The intra-experimental variation in the cell-type distribution was probably a major factor contributing to the observation of a significant interaction between the exposure order and the electric field in some data sets (Table 4).

The rationale for suggesting that there might be a significant variation in the sample-to-sample cell-type distribution is based on two factors. First, the time required to expose all of the samples in a given experiment

TABLE 4A

10 μsec Mitogenicity Data
Results of Type IV[13] Analysis of Variance Tests

Model:[14] ln (APC) = V (error) + EF + EXPT

Concentration of LPS (μgm/ml)	R^2	Source	Degrees of Freedom	F	P
0	0.6242	EF	11	1.29	0.2770
		EXPT	4	9.19	0.0001
200	0.9326	EF	10	14.59	0.0001
		EXPT	2	66.40	0.0001
400	0.6848	EF	11	3.05	0.0071
		EXPT	4	7.27	0.0003
800	0.8905	EF	10	9.64	0.0001
		EXPT	2	37.07	0.0001

[13] In Type IV analysis of variance the variable being tested is added to the model last. F and P values for Type IV tests are equivalent to the results of a t-test for testing the hypothesis that the regression parameter equals zero (266).

[14] APC = cpm $^3H/10^5$ cells; EF = effect of the electric field; EXPOS = effect due to the order of exposure; EXPT = effect of experiment-to-experiment variation; V (error) = variation due to error.

TABLE 4B

10 μsec Mitogenicity Data

Results of Type IV Analysis of Variance Tests

(Continued)

Model: ln (APC) = V (error) + EF + EXPOS + EXPT

Concentration of LPS (μgm/ml)	R^2	Source	Degrees of Freedom	F	P
0	0.7595	EF	11	0.87	0.5839
		EXPOS	11	1.02	0.4625
		EXPT	4	9.09	0.0002
200	0.9736	EF	10	11.92	0.0002
		EXPOS	11	1.56	0.2373
		EXPT	2	66.38	0.0001
400	0.7958	EF	11	2.11	0.0705
		EXPOS	11	0.99	0.4875
		EXPT	4	7.45	0.0008
800	0.9460	EF	10	7.27	0.0015
		EXPOS	11	1.03	0.4826
		EXPT	2	29.34	0.0001

(Continued)

TABLE 4C

10 μsec Mitogenicity Data

Results of Type IV Analysis of Variance Tests

(Continued)

Model: ln (APC) = V (error) + EF + EXPOS + EF*EXPOS + EXPT

Concentration of LPS (μgm/ml)	R^2	Source	Degrees of Freedom	F	P
0	0.9481	EF	11	0.96	0.5593
		EXPOS	11	0.97	0.5546
		EF*EXPOS	15	1.21	0.4492
		EXPT	4	3.28	0.1124
200	0.9989	EF	10	76.16	0.0022
		EXPOS	11	5.55	0.0924
		EF*EXPOS	8	8.98	0.0489
		EXPT	2	110.87	0.0015
400	0.9515	EF	11	2.78	0.1344
		EXPOS	11	1.57	0.3240
		EF*EXPOS	15	1.07	0.5136
		EXPT	4	2.74	0.1493
800	0.9941	EF	10	17.85	0.0184
		EXPOS	11	1.12	0.5247
		ET*EXPOS	8	3.05	0.1947
		EXPT	2	11.37	0.0348

ranged from 1/2 to 2 1/2 hours, with most of the 2 μsec experiments lasting 1 1/2 or 2 hours. During this exposure period the cells were maintained at a very high cell concentration (0.5-1.0 x 10^8 cells/ml) in an isotonic sucrose solution, undesirable conditions for cell survival. One consequence of this situation is the probable occurrence of a difference in the overall susceptibility of the cell population to the effects of the electric field over the course of an experiment. In addition, the well-established differential adsorption of the various leukocytes to plastic surfaces (e.g. the sample test tube) could lead to differential loss of the component cells from the sample resulting in a significant change in the monocyte:B:T cell ratios during an experiment. This effect could be amplified further if there is, as seems likely, a differential susceptibility to the electric field between cell types. Thus, in retrospect, significant intra-experimental differences in both the cell-type distribution and the overall susceptibility of cells to pulsed electric fields seem likely. The effect of these processes on the variability of the results of the mitogenic assays was probably increased further by the randomization of the exposure order.

Another factor that would have obscured the detrimental effects of 2 μsec high field strength pulses is the apparent ability of mouse spleen cells to repair the

injury resulting from such exposures when incubated at 37°C for as little as two hours. This ability to reverse the damage caused by an electrical impulse is indicated by a decrease in Trypan blue uptake by mouse spleen lymphocytes diluted with culture medium (RPMI 1640) and incubated for 2 hours at 37°C as compared with the dye uptake by cells maintained at room temperature (22°C) following exposure to a pulsed electric field. The result, reported above, is consistent with observations made on red blood cells (73,80) and thymocytes (75). This result also suggests that the level of damage produced by a 2 μsec pulse is generally less severe than that produced by a 10 μsec pulse. This is in agreement with observations made on the dependence of field-induced loss of intracellular potassium. Further, these results also suggest that the pulse duration threshold for irreversible cell damage from transient electric fields is in the range of 2 to 10 μsec.

The high level of variability encountered in the mitogenic assays in this investigation is unfortunate in that it leaves a number of questions unanswered. This is particularly true in regard to the possible stimulation of lymphocyte proliferation by the electrical field, per se, or the enhancement of the mitogenic response in cells exposed to an electrical pulse. The observations presented here do not show a statistically significant enhancement

of the mitogen stimulated lymphocyte proliferation in cells exposed to pulsed electrical fields. At the same time, the persistent occurrence of individual samples which incorporate tritiated thymidine to levels up to ten times in excess of the control response levels makes it impossible to rule out either an enhancement of the mitogenic response or stimulation of lymphocyte proliferation by the electric field. The high exposed:control response ratios are generally associated with samples having relatively low levels of tritium incorporation. Such low responses were encountered in experiments cultured for long periods (i.e. >84 hours) (Fig. 17), samples in which no mitogen was used, and the initial 2 μsec mitogenicity experiment.[12] The results of these experiments suggest that the exposure to a single electric pulse at sublethal field strengths has a growth stimulating effect on some parts of the mouse spleen cell population. This is consistent with observations reported on lymphocytes exposed to microwave radiation during culture (223), and with the well-established growth stimulating effects of low voltage DC and repetitively pulsed electric fields on bone, cartilage, nerves, nucleated red blood cells, etc. (18-24). The existence of

---

12. This experiment was not included in the 2 μsec data pool due to its low level response, the relatively flat mitogen concentration curve, and a culture period in excess of 72 hours.

such an effect would also be strong support for the idea that a change in the transmembrane potential functions as a trigger mechanism for cell proliferation.

To date, all of the studies on the effects of pulsed electric fields have been interpreted in terms of alterations in the cell membrane's semi-permeable properties. This interpretation is based on the classical model of a cell as a membrane surrounded solution. It should be pointed out that all of the previous results and those obtained in this study are also completely compatible with more recent cell models that ascribe a less significant roll to the cell membrane. These models include Ling's association-induction model (164,165) and Cope's solid state physics model (260-262). All of these models view the cell interior as being highly structured with a major portion of the water in an oriented state somewhere between that found in ice and the random disorder of liquid water. These models also consider the K^+ and Na^+ ions in the cell as existing as "bound" or "in association" with the proteins and the other macromolecular constituents of the cell. If these models are correct then the effect of a pulsed electric field would be due to the reorientation of this macromolecular-water-ion complex in the cell's interior. The membrane would retain its electrical properties so it would effectively insulate the cell interior from the detrimental effects of the electric

field until dielectric breakdown occurred. After that point the electric field would exert its effects on the cytoplasmic constituents. This senario provides an adequate explanation for the results presented above and for the observation on erythrocytes and other cell types (1-6).

Although the theories of Ling, Cope, and others have not been widely accepted, a number of factors suggest that the effects of pulsed electric fields should be considered in terms of these models as well as the classic cell model. Certainly, there is little doubt that the picture of the cell as a membranous bag of enzymes, ions, etc. dissolved in an aqueous solution is greatly over-simplified. The use of electron microscopy and other modern techniques in the study of molecular and cellular physiology over the last two decades has continued to reveal the cell to be a complex, highly organized entity, and new evidence is continually being accumulated suggesting a more realistic cell model might be of use in gaining better insight into the functioning of cells. For example, the recent investigation of salt and water balance in lymphocytes by Negendank et al. (150,159-163) has been interpreted in terms of the association-induction model. In other areas of investigation, Zimmermann et al. (75) have recently observed that the exposure of thymocytes to a pulsed electric field in excess of 0.5 μsec resulted in

alterations to the cells' internal components that were detectable in electron micrographs, and Kaibara and Tsong (84) have reported that the exposure of sickle human erythrocytes to pulsed electric fields with amplitudes below the membrane breakdown point facilitated the oxygenation and unsickling of these cells. Taken together, these three studies suggest that the effects of pulsed electric fields would benefit from consideration of a more realistic cell model. As previously indicated, a senario of electric field induced cell damage in terms of an "association-induction" type model is very compatible with both the results of this study and with the results of earlier studies (1-6). In addition, such an interpretation provides a more satisfying explanation of the recent observations of Zimmerman et al. (75) and those of Kiabara and Tsong than do simple membrane models.

Unfortunately, these results and those obtained in this study on the effects of pulsed electric fields do not constitute a definitive proof for any of the newer models, nor disproof of the classic model. Indeed, a definitive proof of any of these models seems unlikely, as one is left with only "supportive" evidence on which to base his conjectures. As the truth is probably somewhere in between the two extremes, it seems worthwhile to consider experimental observations from both points of view. This practice is likely to give better insight

into the actual mechanisms of cell perturbation by physical treatments (e.g. pulsed electric field), and may also add support in favor of one of the alternatives.

Considering the potential implications of studies on the effects of pulsed electric fields on cells, particularly the possible initiation of cell proliferation, to the general fields of molecular and cellular physiology, and the importance of these issues in the fields of public and occupational safety, it would seem prudent to extend this investigation in directions that address this specific problem in a definitive manner. On the basis of this investigation and information from related fields of inquiry, the following suggestions are offered as the basis of a more definitive investigation.

As indicated in the Introduction, it is difficult to establish necessary and sufficient conditions for initiating a complex process such as cell proliferation, and much time and resources have been expanded without producing any useful result. This is primarily due to the failure of most investigators to consider the true complexity of the problem when designing their experimental protocol. In the investigation of the effects of a pulsed electric field on cell growth and proliferation, it will be necessary to obtain data on the electric field, information on changes in the ionic constituents of the cells, including monitoring of size changes, and

general morphological data, in addition to monitoring the actual cell proliferation. Consequently, this investigation will require a complex protocol that could not be effectively executed without the cooperation of two or three people. There are simply too many variables to be monitored and controlled. While this involvement of several individuals is contrary to some traditions, it would seem that this situation will become the rule rather than the exception if the more complex questions confronting the biological sciences are to be investigated successfully.

One of the controls that the proposed study should include is the cell-type distribution, initially, at the time of exposure, and at various points throughout the course of the experiment. Although this will be tedious and time consuming, the information provided by these determinations will be necessary to correct and interpret the data from the other parts of the experiment. It may, for instance, provide some indication of differential sensitivity of various cell types to the effects of a pulsed electric field. The results of these differential cell counts in the initial experiments may also indicate if it would be practical to work with a purified cell population.

It is suggested that cell potassium levels continue to be monitored as this is a well-established indicator

of cell injury. In addition, two other assays should be explored as possible monitors of cell damage. One is the phase contrast technique developed by Schrek (238-240) to monitor the effects of noxious agents and various physical treatments on cells. This technique might be supplemented or modified by using multiple nuclear stains of fixed samples (263). The second technique that should be explored as an indicator of cell viability and vitality is the cell's ability to regulate its volume under hypotonic conditions (151,198). Buckhold Shank has suggested this as a means of accessing a cell's metabolic status (198). The use of either or both of these techniques would facilitate the determination of the extent, the nature, and the time course of any subcellular damage produced by the pulsed electric field. These techniques would also provide information regarding possible repair processes elicited from the exposed cells.

The combining of these various techniques to monitor the cell concentration, cell-type distribution and physical status of the cell population as a whole together with the measurement of cell growth and proliferation should provide the information required for the successful execution of this investigation.

In measuring cell stimulation it may be informative to incubate part of each sample with one optimal concentration of mitogen, one clearly suboptimal concentration

of mitogen and no mitogen. Initial studies should be conducted to determine the exact nature of the time response of the cells to mitogenic and electrical stimulation. In measuring cell proliferation serious consideration should be given to the use of ^{125}I-5' iododeoxyuridine (^{125}IdU) in place of tritiated thymidine. There are a number of advantages to using the relatively short lived gamma emitting isotope including reduced cell radiation damage and reduced waste disposal problems. The major advantage in using ^{125}IdU is the ability to count the samples in a solid scintillation counter eliminating the disadvantages of liquid scintillation counting. Unfortunately, the extent of the problems encountered in the liquid scintillation counting of tritium that is associated with macromolecular DNA or protein are not fully appreciated by most investigator (264). The problems that are likely to effect the results from such an assay using ^{3}H include chemo- and photoluminescence, static build-up on vials, inter-sample differences and intra-sample variation in quenching, and poor and changing counting geometry. The latter problems and the intra-sample variation in quenching are the result of the serious, but little recognized, problem of the slow adsorption of macromolecular DNA and protein to the walls of the scintillation vials (264). This is probably one of the unrecognized contributions to the high degree of

variability associated with mitogenic assays. Another modification of the assay for cell proliferation that should be thoroughly explored is the use of serum-free media (265). This would be particularly important in accessing the possibility of cell stimulation by the electric field, per se, as serum is known to produce some lymphocyte proliferation by itself (252,264).

The study proposed here would be an ambitious undertaking; however, it seems unlikely that a simpler investigation would provide a definitive answer to the question of the possible stimulation of lymphocytes by a pulsed electric field.

The results reported above indicate that exposure of mouse spleen lymphocytes to a high voltage electrical pulse is capable of producing irreversible cell damage leading to cell death. The threshold for such irreversible cell injury is dependent on both the strength of the electric field and the duration of the pulse. For a 10 μsec electrical pulse, this threshold occurs between 2.0 and 2.4 kV/cm, as indicated by a significant decrease in the response of exposed cells to mitogenic stimulation and a statistically significant efflux of intracellular K^+. The results of this study suggest that sublethal electrical pulses may stimulate the proliferation of mouse spleen lymphocytes, a possibility that should be explored more thoroughly.

BIBLIOGRAPHY

1. Sale, A.J.H. and Hamilton, W.A.: Effects of high electric fields on micro-organisms I. Killing of bacteria and yeasts. Biochim. Biophys Acta 148: 781-788, 1967.

2. Hamilton, W.A. and Sale, A.J.H.: Effects of high electric fields on micro-organisms. II. Mechanism of action of the lethal effect. Biochim. Biophys. Acta 148:789-800, 1967.

3. Sale, A.J.H. and Hamilton, W.A.: Effects of high electric fields on micro-organisms. III. Lysis of erythrocytes and protoplasts. Biochim. Biophys. Acta 163:37-43, 1968.

4. Zimmermann, U., Pilwat, G., Beckers, F. and Riemann, F.: Effects of external electrical fields on cell membranes. Bioelectrochem. Bioenerg. 3:58-83, 1976.

5. Kinosita, K., Jr. and Tsong, T.Y.: Hemolysis of human erythrocytes by a transient electric field. Proc. Natl. Acad. Sci. (USA) 74:1923-1927, 1977.

6. Cleary, S.F., Hoffman, R. and Liu, L.M.: Investigation of the biological effects of pulsed electrical fields. Report No. 2, Contract No. N00014-75-C-0334, Office of Naval Research, Arlington, VA, (Springfield, VA: NTIS, 1977).

7. Cleary, S.F.: Biological effects of microwave and radiofrequency. CRC Crit. Rev. Environ. Control 7: 121-166, 1977.

8. Baranski, S. and Czerski, P.: Biological Effects of Microwaves, (Stroudsburg, PA: Dowden, Hutchinson and Ross, Inc., 1976).

9. Stuchly, M.A.: Interaction of radiofrequency and microwave radiation with living systems: A review of mechanisms. Radiat. Environm. Biophys. 16:1-14, 1979.

10. Magnien, M. (Ed.): Recherches sur les effets biologiques des champs electrique et magnetique. Rev. Gen. Electricite no. special:1-101, Juillet, 1976.

11. Adey, W.R.: Tissue interactions with nonionizing electromagnetic fields. Physiol. Rev. 61:435-514, 1981.

12. Pudenz, R.H., Agnew, W.F., Yuen, T.G.H., Bullara, L.A., Jacques, S. and Shelden, C.H.: Adverse effects of electrical energy applied to the nervous system. Appl. Neurophysiol. 40:72-87, 1977.

13. Takashima, S., Onaral, B. and Schwan, H.P.: Effects of modulated RF energy on the EEG of mammalian brains. Radiat. Environm. Biophys. 16:15-27, 1979.

14. Artz, C.P.: Electrical injury. in: Burns: A Team Approach, Artz, C.P., Moncrief, J.A. and Pruitt, Jr., B.A. (Eds.), (Philadelphia: W.B. Saunders Co., 1979), pp. 351-362.

15. McDougal, W.S., Slade, C.L. and Pruitt, B.A., Jr. Electrical injuries, in: Manual of Burns, (New York: Springer-Verlag, 1978), pp. 93-98.

16. Liboff, A.R. and Rinaldi, R.A. (Eds.): Electrically mediated growth mechanisms in living systems. Ann. N.Y. Acad. Sci. 238:1-593, 1974.

17. Jaffe, L.F. and Nuccitelli, R.: Electrical controls of development. Ann. Rev. Biophys. Bioeng. 6:445-476, 1977.

18. Pilla, A.A.: Mechanisms of electrochemical phenomena in tissue repair and growth. Bioelectochem. Bioenerg. 1:227-243, 1974.

19. Pilla, A.A.: Electrochemical information transfer at cell surfaces and junctions--Application to the study and manipulation of cell regulation. in: Bioelectrochemistry; Proc. U.S.-Australia Joint Seminar on Bioelectrochemistry, Pasadena, CA, July 9-12, 1979, Keyser, H.K. and Gutmann, F. (Eds.), (New York: Plenum Press, 1979), pp. 353-396.

20. Popp, F.A., Becker, G., König, H.L. and Peschka, W. (Eds.): Electromagnetic Bio-Information, (Baltimore: Urban and Schwarzenberg, 1979).

21. Becker, R.O. and Pilla, A.A.: Electrochemical mechanisms and the control of biological growth processes. in: Modern Aspects of Electrochemistry, (New York: Plenum Press, 1975), pp. 289-338.

22. Smith, S.D., Thomas, C.L. and Frasch, S.F.: Lanthanum inhibition of electrically induced de-differentiation in frog erythrocytes. Bioelectrochem. Bioenerg. 5:177-184, 1978.

23. Burny, F., Herbst, E. and Hinsenkamp, M. (Eds.): Electric Stimulation of Bone Growth and Repair, (New York: Springer-Verlag, 1978).

24. Brighton, C.T., Black, J. and Pollack, S.R.: Electrical Properties of Bone and Cartilage; Experimental Effects and Clinical Applications (New York: Grune & Stratton, 1979).

25. Har-Kedar, I. and Bleehen, N.M.: Experimental and clinical aspects of hyperthermia applied to the treatment of cancer with special reference to the role of ultrasonic and microwave heating. Advan. Radiat. Biol. 6:229-266, 1976.

26. Hahn, G.M.: The use of microwaves for the hyperthermic treatment of cancer: Advantages and disadvantages. Photochem. Photobiol. Rev. 3:277-301, 1978.

27. Lauf, P.K.: Membrane immunological reactions and transport. in: Membrane Transport in Biology, I, Concepts and Models, Tosteson, D.C. (Ed.), (New York: Springer-Verlag, 1978), pp. 291-348.

28. Steinhardt, R.A., Lundin, L. and Mazia, D.: Bioelectric responses of the echinoderm egg to fertilization. Proc. Natl. Acad. Sci. (USA) 68:2426-2430, 1971.

29. Steinhardt, R.A. and Winkler, M.M.: The ionic hypothesis of cell activation at fertilization. in: The Molecular Basis of Immune Cell Function. Proc. 13th Internatl. Leucocyte Culture Conf. Kaplin, J.G. (Ed.), (Amsterdam: Elsevier/North Holland Biomedical Press, 1979), pp. 29-37.

30. Riddle, V.G.H., Rossow, P.W., Boorstein, R.J., Addonizio, M.L. and Pardee, A.B.: Can a fibroblast teach a lymphocyte anything useful? in: Ibid., pp. 11-27.

31. Kaplan, J.G.: Membrane cation transport and the control of proliferation of mammalian cells. Ann. Rev. Physiol. 40:19-41, 1978.

32. Kaplan, J.G. and Owens, T.: Activation of lymphocytes of man and mouse: Monovalent cation fluxes. Ann. N.Y. Acad. Sci. 339:191-200, 1980.

33. Leffert, H.L. (Ed.): Growth Regulation by Ion Fluxes. Ann. N.Y. Acad. Sci. 339:1-335, 1980.

34. Jaffe, L.F.: Control of development by ionic currents. in: Membrane Transduction Mechanisms, Cone, R.A. and Dowling, J.E. (Eds.), (New York: Raven Press, 1979).

35. Neumann, E. and Rosenheck, K: Permeability changes induced by electric impulses in vesicular membranes. J. Membr. Biol. 10:279-290, 1972.

36. Rosenheck, K., Lindner, P. and Pecht, I.: Effect of electric fields on light-scattering and fluorescence of chromaffin granules. J. Membr. Biol. 20:1-12, 1975.

37. Turnbull, R.J.: An alternate explanation for the permeability changes induced by electrical impulses in vesicular membranes. J. Membr. Biol. 14:193, 1973.

38. Neumann, E. and Rosenheck, K.: Potential differences across vesicular membranes. J. Membr. Biol. 14:194-196, 1973.

39. Kachel, V.: Electrical resistance pulse sizing (Coulter Sizing). in: Flow Cytometry and Sorting, Melamed, M.R., Mullaney, P.F., and Mendelson, M.L. (Eds.), (New York: Wiley, 1979) pp.61-104.

40. Harvey, R.J.: Measurement of cell volumes by electric sensing zone instruments. Methods in Cell Physiol. 3:1-23, 1968.

41. Ur, A. and Lushbaugh, C.C.: Some effects of electrical fields on red blood cells with remarks on electronic red cell sizing. Brit. J. Haemat. 15:527-538, 1968.

42. Grover, N.B., Naaman, J., Ben-Sasson, S. and Doljanski, F: Electrical sizing of particles in suspensions. I. Theory, Biophys. J. 9:1398-1414, 1969.

43. Grover, N.B., Naaman, J., Ben-Sasson, S., Doljanski, F. and Nadav, E.: Electrical sizing of particles in suspensions: II. Experiments with rigid spheres. Biophys. J. 9:1415-1425, 1969.

44. Grover, N.B., Naaman, J., Ben-Sasson, S. and Doljanski, F.: Electrical sizing of particles in suspensions: III. Rigid spheroids and red blood cells. Biophys. J. 12:1099-1116, 1972.

45. Ben-Sasson, S., Patinkin, D., Grover, N.B. and Doljanski, F.: Electrical sizing of particles in suspensions: IV. Lymphocytes. J. Cell. Physiol. 84:205-214, 1974.

46. Von Behrens, W. and Edmondson, S.: Comparison of techniques improving the resolution of standard coulter cell sizing systems. J. Histochem. Cytochem. 24:247-256, 1976.

47. Kachel, V.: Basic principles of electrical sizing of cells and particles and their realization in the new instrument "Metricell." J. Histochem. Cytochem. 24:211-230, 1976.

48. Leif, R.C., Guarino, V. and Lefkove, N.: The automated multiparameter analyzer for cells (AMAC) IIA, A true bridge circuit coulter-type electronic cell volume transducer. J. Histochem. Cytochem. 27:225-233, 1979.

49. Zimmermann, U., Schulz, J. and Pilwat, G.: Transcellular ion flow in Escherichia coli B and electrical sizing of bacterias. Biophys. J. 13:1005-1013, 1973.

50. Zimmermann, U., Pilwat, G. and Riemann, F.: Preparation of erythrocyte ghosts by dielectric breakdown of the cell membrane. Biochim. Biophys. Acta 375:209-219, 1975.

51. Zimmermann, U., Pilwat, G., Holzopfel, C. and Rosenheck, K.: Electrical hemolysis of human and bovine red blood cells. J. Membr. Biol. 30:135-152, 1976.

52. Riemann, F., Zimmermann, U., and Pilwat, G.: Release and uptake of haemoglobin and ions in red blood cells induced by dielectric breakdown. Biochim. Biophys. Acta 394:449-462, 1975.

53. Pilwat, G., Zimmermann, U. and Riemann, F.: Dielectric breakdown measurements of human and bovine erythrocyte membrane using benzyl alcohol as a probe molecule. Biochim Biophys. Acta 406:424-432, 1975.

54. Zimmermann, U., Riemann, F. and Pilwat, G.: Enzyme loading of electrically homogeneous human red blood cell ghosts prepared by dielectric breakdown. Biochim. Biophys. Acta 436:460-474, 1976.

55. Schneeweiss, F., Zimmermann, U. and Saleemuddin, M.: Preparation of uniform haemoglobin free human erythrocyte ghosts in isotonic solution. Biochim. Biophys. Acta 466:373-378, 1977.

56. Zimmermann, U., Pilwat, G. and Riemann, F.: Dielectric breakdown of cell membranes. in: Membrane Transport in Plants, Zimmermann, U. and Dainty, J. (Eds.), (New York: Springer-Verlag, 1974), pp. 146-153.

57. Zimmermann, U.: Cell turgor pressure regulation and turgor pressure--Mediated transport processes. in: Integration of Activity in the Higher Plant, Jennings, D. (Ed.), (Cambridge: University Press, 1977); Symp. Soc. Exp. Biol. 31:117-154, 1977.

58. Jeltsch, E. and Zimmermann, U.: Particles in a homogeneous electric field: A model for the electrical breakdown of living cells in a coulter counter. Bioelectrochem. Bioenerget. 6:349-384, 1979.

59. Zimmermann, U., Groves, M., Schnabl, H. and Pilwat, G.: Development of a new coulter counter system: Measurment of the volume, internal conductivity and dielectric breakdown voltage of a single guard cell protoplast of Vicia faba. J. Membr. Biol. 52:37-50, 1980.

60. Zimmermann, U., Pilwat, G. and Riemann, F.: Dielectric breakdown of cell membranes. Biophys. J. 14:881-899, 1974.

61. Coster, H.G.L. and Zimmermann, U.: Dielectric breakdown in the membranes of Valonia utricularis. The role of energy dissipatation. Biochim. Biophys. Acta 382:410-418, 1975.

62. Coster, H.G.L. and Zimmermann, U.: Direct demonstration of dielectric breakdown in the membranes of Valonia utricularis. Z. Naturforsch. 30c:77-79, 1975.

63. Coster, H.G.L. and Zimmermann, U.: The mechanisms of electrical breakdown in the membranes of Valonia utricularis. J. Membr. Biol. 22:73-90, 1975.

64. Benz, R., Beckers, F. and Zimmermann, U.: Reversible electric breakdown of lipid bilayer membranes: A charge-pulse relaxation study. J. Membr. Biol. 48:181-204, 1979.

65. Benz, R. and Zimmermann, U.: Pulse-length dependence of the electrical breakdown in lipid bilayer membranes. Biochim. Biophys. Acta 597:637-642, 1980.

66. Benz, R. and Zimmermann, U.: The resealing process of lipid bilayers after reversible electrical breakdown. Biochim. Biophys. Acta 640:169-178, 1981.

67. Coster, H.G.L.: A quantitative analysis of the voltage-current relationships of fixed charge membranes and the associated property of "punch-through." Biophys. J. 5:669-686, 1965.

68. Crowley, J.M.: Electrical breakdown of bimolecular lipid membranes as an electromechanical instability. Biophys. J. 13:711-724, 1973.

69. Groves, M.R.: Application of the electrical sizing principle of coulter to a new multiparameter system. IEEE Trans. Biomed. Eng. BME-27:364-369, 1980.

70. Liburdy, R.P.: Increased dielectric breakdown potential of spleen lymphocytes from mice undergoing allograft rejection. Fed. Proc. 38:1431, 1979.

71. Pilwat, G., Washausen, P., Klein, J. and Zimmermann, U.: Immobilization of human red blood cells. Z. Naturforsch. c35:352-356, 1980.

72. Zimmermann, U. and Pilwat, G.: Organ-specific application of drugs by means of cellular systems. Z. Naturforsch c31:732-736, 1976.

73. Zimmermann, U., Pilwat, G. and Esser, B.: The effect of encapsulation in red blood cells on the distribution of methotrexate in mice. J. Clin. Chem. Clin. Biochem. 16:135-144, 1978.

74. Zimmermann, U., Pilwat, G. and Vienken, J.: Erythrocytes and lymphocytes as drug carrier systems: Techniques for entrapment of drugs in living cells. in: Cancer Chemo and Immunopharmacology; 2. Immunopharmacology, Relations and General Problems, Mathé, G. and Muggia, F.M., (Eds.), (Berlin: Springer-Verlag, 1980); Rec. Results Cancer Res. 75:252-259, 1980.

75. Zimmermann, U., Vienken, J. and Pilwat, G.: Development of drug carrier systems: Electrical field induced effects in cell membranes. Bioelectrochem. Bioenerget. 7:553-574, 1980.

76. Tsong, T.Y. and Kingsley E: Hemolysis of human erythrocyte induced by a rapid temperature jump. J. Biol. Chem. 250:786-789, 1975.

77. Tsong, T.Y., Tsong, T-T., Kingsley, E. and Siliciano, R.: Relaxation phenomena in human erythrocyte suspensions. Biophys. J. 16:1091-1104, 1976.

78. Kinosita, K., Jr. and Tsong, T.Y.: Formation and resealing of pores of controlled sizes in human erythrocyte membrane. Nature 268:438-441, 1977.

79. Kinosita, K., Jr., and Tsong, T.Y.: Voltage-induced pore formation and hemolysis of human erythrocytes. Biochim. Biophys. Acta 471:227-242, 1977.

80. Kinosita, K., Jr. and Tsong, T.Y.: Survival of sucrose-loaded erythrocytes in the circulation. Nature 272:258-260, 1978.

81. Kinosita, K., Jr. and Tsong, T.Y.: Voltage-induced changes in the conductivity of erythrocyte membranes. Biophys. J. 24:373-375, 1978.

82. Kinosita, K., Jr. and Tsong, T.Y.: Voltage-induced conductance in human erythrocyte membranes. Biochim. Biophys. Acta 554:479-497, 1979.

83. Teissie, J. and Tsong, T.Y.: Evidence of voltage-induced channel opening in Na/K ATPase of human erythrocyte membrane. J. Membrane Biol. 55:133-140, 1980.

84. Kaibara, M. and Tsong, T.Y.: Voltage pulsation of sickle erythrocytes enhances membrane permeability to oxygen. Biochim. Biophys. Acta 595:146-150, 1980.

85. Katchalsky, A. ad Curran, P.F.: Nonequilibrium Thermodynamics in Biophysics, (Cambridge, MA: Harvard University Press, 1965), pp. 181-198.

86. Cleary, S.F.: Investigation of the biological effects of pulsed electric fields, Report No. 3, Contract No. N00014-75-C-0334, Office of Naval Research, Arlington, VA, (Springfield, VA: NTIS, 1978).

87. Gauger, B. and Bentrup, F.W.: A study of dielectric membrane breakdown in the Fucus egg. J. Membrane Biol. 48:249-264, 1979.

88. Sargent, D.F.: Voltage jump/capacitance relaxation studies of bilayer structure and dynamics. J. Membrane Biol. 23:227-247, 1975.

89. DeMaeyer, L.C.M.: Electric field methods. Methods Enzymol. 16:80-118, 1969.

90. Eyring, E.M.: The electric field jump relaxation method. in: Chemical and Biological Applications of Relaxation Spectrometry, Wyn-Jones, E. (Ed.), (Dordrecht-Holland: D. Reidel, 1975), pp. 85-90.

91. Bernasconi, C.F.: Relaxation Kinetics, (New York: Academic Press, 1976).

92. Gavach, C., Seta, P., Sandeaux, R. and Cros, D.: Relaxation electrical study of hydrophobic ion transport across lipid bilayer membranes. in: Electrical Phenomena at the Biological Membrane Level, Roux, E. (Ed.), (Amsterdam: Elsevier, 1977), pp. 419-429.

93. Sargent, D.F.: An apparatus for measurement of very small membrane relaxation currents. Anal. Biochem. 70:100-109, 1976.

94. Benz, R. and Läuger, P.: Kinetic analysis of carrier-mediated ion transport by the charge-pulse technique. J. Membrane Biol. 27:171-191, 1976.

95. White, S.H.: Comments on "Electrical breakdown of bimolecular lipid membranes as an electromechanical instability." Biophys. J. 14:155-158, 1974.

96. White, S.H.: Analysis of the torus surrounding planar lipid bilayer membranes. Biophys. J. 12:432-445, 1972.

97. Benz, R. and Janko, K.: Voltage-induced capacitance relaxation of lipid bilayer membranes: Effects of membrane composition. Biochim. Biophys. Acta 455: 721-738, 1976.

98. Bamberg, E. and Benz, R.: Voltage-induced thickness changes of lipid bilayer membranes and the effect of an electric field on gramicidin A channel formation. Biochim. Biophys. Acta 426:570-580, 1976.

99. Parsegian, A.: Energy of an ion crossing a low dielectric membrane: Solutions of four relevant electrostatic problems. Nature 221:844-846, 1969.

100. Gurney, R.W.: Ionic Processes in Solution, (New York: Dover Publications, 1962).

101. Requena, J., Haydon, D.A. and Hladky, S.B.: Lenses and the compression of black lipid membranes by an electric field. Biophys. J. 15:77, 1975.

102. Evans, E.A. and Hochmuth, R.M.: Mechanochemical properties of membranes. Curr. Top. Membrane Transport 10:1-64, 1978.

103. Evans, E.A. and Simon, S.: Mechanics of electro-compression of lipid bilayer membranes. Biophys. J. 15:850-852, 1976.

104. Chizmadzhev, Yu. A. and Abidor, I.G.: Bilayer lipid membranes in strong electric fields. Bioelectrochem. Bioenerg. 7:83-100, 1980.

105. Benz, R., Fröhlich, O., Läuger, P. and Montal, M.: Electrical capacity of black lipid films and of lipid bilayers made from monolayers. Biochim. Biophys. Acta 394:323-334, 1975.

106. White, S.H., Petersen, D.C., Simon, S. and Yafuso, M.: Formation of planer bilayer membranes from lipid monolayers. A Critique. Biophys. J. 16:481-490, 1976.

107. Zimmermann, U.: Physics of Turgor-and osmoregulation. Ann. Rev. Plant. Physiol. 29:121-148, 1978.

108. Zimmermann, U. and Scheurich, P.: Fusion of Avena Sativa mesophyll cell protoplasts by electrical breakdown. Biochim. Biophys. Acta 641:160-165, 1981.

109. Zimmermann, U., Pilwat, G., Péqueux, A. and Gilles, R.: Electro-mechanical properties of human erythrocyte membranes: The pressure-dependence of potassium permeability. J. Membrane Biol. 54:103-113, 1980.

110. Zimmermann, U. and Hüsken, D.: Turgor pressure and cell volume relaxation in Halicystis parvula. J. Membrane Biol. 56:55-64, 1980.

111. Zimmermann, U. and Benz, R.: Dependence of the electrical breakdown voltage on the charging time in Valonia utricularis. J. Membrane Biol. 53:33-43, 1980.

112. Abidor, I.G., Arakelyan, V.B., Chernamordik, L.V., Chizmadzhev, Yu.A., Pastushenko, V.F. and Tarasevich, M.R.: Electric breakdown of bilayer lipid membranes. I. The main experimental facts and their qualitative discussion. Bioelectrochem. Bioenerg. 6:37-52, 1979.

113. Pastushenko, V.F., Chizmadzhev, Yu.A. and Arakelyan, V.B.: Electric breakdown of bilayer lipid membranes. II. Calculation of the membrane lifetime in the steady-state diffusion approximation. Bioelectrochem. Bioenerg. 6:53-62, 1979.

114. Chizmadzhev, Yu.A., Arakelyan, V.B. and Pastushenko, V.F.: Electric breakdown of bilayer lipid membranes. III. Analysis of possible mechanisms of defect origination. Bioelectrochem. Bioenerg. 6:63-70, 1979.

115. Pastushenko, V.F., Chizmadzhev, Yu.A. and Arakelyan, V.B.: Electric breakdown of bilayer lipid membranes. IV. Consideration of the kinetic stage in the case of the single-defect membrane. Bioelectrochem. Bioenerg. 6:71-79, 1979.

116. Arakelyan, V.B., Chizmadzhev, Yu.A. and Pastushenko, V.F.: Electric breakdown of bilayer lipid membranes. V. Consideration of the kinetic stage in the case of the membrane containing an arbitrary number of defects. Bioelectrochem. Bioenerg. 6:81-87, 1979.

117. Pastushenko, V.F., Arakelyan, V.B. and Chizmadzhev, Yu.A.: Electric breakdown of bilayer lipid membranes. VI. A stochastic theory taking into account the processes of defect formation and death: Membrane lifetime distribution function. Bioelectrochem. Bioenerg. 6:89-95, 1979.

118. Pastushenko, V.F., Arakelyan, V.B. and Chizmadzhev, Yu.A.: Electric breakdown of bilayer lipid membranes. VII. A stochastic theory taking into account the processes of defect formation and death: Statistical properties. Bioelectrochem. Bioenerg. 6:97-104, 1979.

119. Sugár, I.P.: A theory of the electric field-induced phase transition of phospholipid bilayers. Biochim. Biophys. Acta 556:72-85, 1979.

120. Douglas, S.D.: Human lymphocyte growth in vitro: morphologic, biochemical, and immunologic significance. Internat. Rev. Exptl. Pathol. 10:41-114, 1971.

121. Elves, M.W.: The Lymphocytes, 2nd ed. (Chicago: Year Book Medical Publisher, 1972).

122. Jerne, N.K.: The common sense of immunology. Cold Spring Harbor Symp. Quant. Biol. 41(pt.1):1-4, 1976.

123. Cline, M.J.: The White Cell, (Cambridge, MA: Harvard University Press, 1975).

124. Bessis, M.: Living Blood Cells and Their Ultrastructure, (New York: Springer-Verlag, 1973).

125. Doljanski, F., Ben-Sasson, S., Reich, M. and Grover, N.B.: Dynamic osmotic behavior of chick blood lymphocytes. J. Cell. Physiol. 84:215-224, 1974.

126. Steen, H.B. and Nielsen, V.: Lymphocyte blastogenesis studied by volume spectroscopy. Scand. J. Immunol. 10:135-143, 1979.

127. Ling, N.R. and Kay, J.F.: Lymphocyte Stimulation, rev. ed., (Amsterdam: North Holland, 1975).

128. Jett, M. and Hickey-Williams, C.: Lymphoid cells. in: Mammalian Cell Membranes, vol. 3, Surface Membranes of Specific Cell Types, Jamieson, G.A. and Robinson, D.M. (Eds.), (London: Butterworths, 1977), pp. 47-88.

129. Katz, D.H. and Benacerraf, B.: The regulatory influence of activated T cells on B cell responses to antigen. Advan. Immunol. 15:1-94, 1972.

130. Glick, B., Chang, T.S. and Jaap, R.G.: The bursa of fabricius and antibody production. Poultry Sci. 35:224, 1956.

131. Good, R.A. and Papermaster, B.W.: Ontogeny and phylogeny of adoptive immunity. Advan. Immunol. 4:1-116, 1963.

132. Strober, S.: Immune function cell surface characteristrics and maturation of B cell subpopulations. Transplant. Rev. 24:84-112, 1975.

133. Mathé, G., Florentin, I. and Simmler, M.-C. (Eds.), Lymphocytes, Macrophages and Cancer, (Berlin: Springer-Verlag, 1976). [Hereafter cited as Rec. Results Cancer Res. 56, 1976].

133a. Stathopoules, G. and Davies, A.J.S.: Immunologic definition of lymphocytes: functions and markers, Rec. Results Cancer Res. 56:17-29, 1976.

134. Catovsky, D.: Morphological recognition of lymphocytes and macrophages. Rec. Results Cancer Res. 56:1-7, 1976.

135. Polliak, A., Lampen, N., Clarkson, B.D., deHarven, E., Bentwich, Z., Siegal, F.P. and Kunkel, H.G.: Identification of human B and T lymphocytes by scanning electron microscopy. J. Exptl. Med. 138: 607-624, 1973.

136. Dantchev, D.: Revised semiology of the different mononuclear cells under scanning electron microscopy. Rec. Results Cancer Res. 56:8-16, 1976.

137. Schlesinger, M.: The lymphocyte membrane. Ser. Haematol. 7:427-445, 1974.

138. Warner, N.L. and McKenzie: Surface antigens and receptors of normal and neoplastic lymphocytes: Nature and distribution. in: The Lymphocyte: Structure and Function, Part II, Marchalonis, J.J. (Ed.), (New York: Marcel Dekker, 1977), pp. 433-492.

139. Janossy, G. and Greaves, M.: Functional analysis of murine and human B lymphocyte subsets. Transplant. Rev. 24:177-236, 1975.

140. Santer, V.B.: The surface coat of lymphocytes. in: The Lymphocyte: Structure and Function, Part II, Marchalonis, J.J. (Ed.), (New York: Marcel Dekker, 1977), pp. 541-564.

141. Mandel, T.E. and Santer, V.B.: Ultrastructure of the lymphocyte surface. in: The Lymphocyte: Structure and Function, Part II, Marchalonis, J.J. (Ed.), (New York: Marcel Dekker, 1977), pp. 511-539.

142. Sharon, N.: Some biological functions ofcell surface sugars. in: Structure and Function of Biomembranes, Yagi, K. (Ed.), (Tokoyo: Japan Scientific Societies Press, 1979), pp. 63-82.

143. Crumpton, J.J. and Snary, D.: Preparation and properties of lymphocyte plasma membrane. Contemp. Top. Molec. Immunol. 3:27-56, 1974.

144. Ji, T.H. and Nicolson, G.L.: Lectin binding and perturbation of the outer surface of the cell membrane induces a transmembrane organizational alteration at the inner surface. Proc. Natl. Acad. Sci. (USA), 71:2212-2216, 1974.

145. DePierre, J.W. and Karnovsky, M.L.: Plasma membranes of mammalian cells. A review of methods for their characterization and isolation. J. Cell Biol. 56:275-303, 1973.

146. Ferber, E. and Resch, K.: Structure and physiological role of lipids in the lymphocyte membrane. in: The Lymphocyte: Structure and Function, Part II, Marchalonis, J.J. (Ed.), (New York: Marcel Dekker, 1977), pp. 593-620.

147. Averdunk, R.: Early changes of "leak flux" and the cation content of lymphocytes by Concanavalin A. Biochem. Biophys. Res. Comm. 70:101-109, 1976.

148. Iversen, J.-G.: Unidirectional K^+ fluxes in rat thymocyte stimulated by concanavalin A. J. Cell. Physiol. 89:267-276, 1976.

149. Lichtman, M.A., Jackson, A.H. and Peck, W.A.: Lymphocyte monovalent cation metabolism: Cell volume, cation content and cation transport. J. Cell. Physiol. 80:383-396, 1972.

150. Negendank, W. and Shaller, C.: Potassium-sodium distribution in human lymphocytes: Description by association-induction hypothesis. J. Cell. Physiol. 98:95-106, 1979.

151. Buckhold Shank, B., Rosenberg, H.M. and Horowitz, C.: Ionic basis of volume regulation in mammalian cells following osmotic shock. J. Cell. Physiol. 82:257-266, 1973.

152. Wilson, D.L. and Manery, J.F.: The permeability of rabbit leukocytes to sodium, potassium, and chloride. J. Cell. Comp. Physiol. 34:493-519, 1949.

153. Cividalli, G. and Nathan, D.N.: Sodium and potassium concentration and transmembrane fluxes in leukocytes. Blood 43:861-869, 1974.

154. Segel, G.B. and Lichtman, M.A.: The effect of method on the measurement of K^+ concentration in PHA-treated human blood lymphocytes. Expt. Cell. Res. 112:95-102, 1978.

155. Negendank, W. and Shaller, C.: The potential for artifact in ion determinations in lectin-treated lymphocytes, in: The Molecular Basis of Immune Cell Function; Proc. 13th Internatl. Leucocyte Culture Conference, Ottawa, Canada, May 22-25, 1979, Kaplan, J.G. (Ed.), (Amsterdam: Elsevier/North Holland Biomedical Press, 1979), pp. 406-407.

156. Metcalfe, J.C., Pozzan, T., Smith, G.A. and Hesketh, T.R.: A calcium hypothesis for the control of cell growth. Biochem. Soc. Symp. 45:1-26, 1980.

157. Freedman, M.H. and Khan, N.R.: A rapid technique for measuring calcium uptake in mitogen-induced T and B lymphocytes. Can. J. Biochem. 57:1344-1350, 1979.

158. Lichtman, A.H., Segel, C.B. and Lichtman, M.A.: An ultrasensitive method for the measurement of human leukocyte calcium:lymphocytes. Clin. Chim. Acta 97:107-121, 1979.

159. Negendank, W. and Shaller, C.: Fast and slow fractions of K^+ flux in human lymphocytes. J. Cell. Physiol. 98:539-552, 1979.

160. Negendank, W. and Shaller C.: A critical temperature transition of K^+-Na^+ exchange in human lymphocytes. J. Cell. Physiol. 103:87-95, 1980.

161. Negendank, W. and Shaller C.: Multiple fractions of sodium exchange in human lymphocytes. J. Cell. Physiol. 104:443-459, 1980.

162. Negendank, W. and Shaller, C.: Simultaneous net accumulation of both K^+ and Na^+ by lymphocytes at 0°C. Biochim. Biophys. Acta 640:368-373, 1981.

163. Negendank, W. and Karreman, G.: Rate of potassium-sodium exchange by human lymphocytes: prediction of the cooperative adsorption model. J. Cell. Physiol. 98:107-112, 1979.

164. Ling, G.N.: A Physical Theory of the Living State: The Association-Induction Hypothesis. (New York: Blaisdell Publishing Co., 1962).

165. Ling, G.N. and Negendank, W.: Do isolated membranes and purified vesicles pump sodium? A critical review and reinterpretation. Perspectives Biol. Med. 23:215-239, 1980.

166. Rink, T.J., Montecucco, C., Hesketh, T.R. and Tsien, R.Y.: Lymphocyte membrane potential assessed with fluorescent probes. Biochim. Biophys. Acta 595:15-30, 1980.

167. Taki, M.: Studies on blastogenesis of human lymphocytes by phytohemagglutinin, with special reference to changes of membrane potential during blastoid transformation. Mie. Med. J. 19:245-262, 1970.

168. Oliveira-Castro, G.M. and Barcinski, M.A.: Calcium-induced uncoupling in communicating human lymphocytes Biochim. Biophys. Acta 352:338-343, 1974.

169. Holian, A., Deutsch, C.J., Holian, S.K. and Daniel, R.P.: Transmembrane potential and pH gradient across human erythrocytes and human lymphocytes. Fed. Proc. 36:826, 1977.

170. Shapiro, H.M., Natale, P.J. and Kamentsky, L.A.: Estimation of membrane potentials of individual lymphocytes by flow cytometry. Proc. Natl. Acad. Sci. (USA) 76:5728-5730, 1976.

171. Kiefer, H., Blume, A.J. and Kaback, H.R.: Membrane potential changes during mitogenic stimulation of mouse spleen lymphocytes. Proc. Natl. Acad. Sci. (USA) 77:2200-2204, 1980.

172. Irimajiri, A., Doida, Y., Hanai, T. and Inouye, A.: Passive electrical properties of cultured murine lymphoblast (L5178Y) with reference to its cytoplasmic membrane, nuclear envelope, and intracellular phases. J. Membrane Biol. 39:209-232, 1978.

173. Nicolini, C.: Normal versus abnormal cell proliferation: A unitary and analytical overview. Cell. Biophys. 2:271-290, 1980.

174. Jeter, J.R., Jr., Cameran, I.L., Padilla, G.M. and Zimmerman, A.M. (Ed): Cell Cycle Regulation (New York: Academic Press, 1978).

175. Papaconstantinou, J. and Rutter, W.J. (Eds.): Molecular Control of Proliferation and Differentiation, (New York: Academic Press, 1978).

176. Oppenheim, J.J. and Rosenstreich, D.L.: Signals regulating in vitro activation of lymphocytes. Prog. Allergy 20:65-194, 1976.

177. Wedner, H.J. and Parker, C.W.: Lymphocyte activation. Prog. Allergy 20:195-300, 1976.

178. Loeb, L.A.: Molecular analysis of lymphocyte transformation, in: Developments in Lymphoid Cell Biology Gottlieb, A.A. (Ed.), (Cleveland, Ohio: CRC Press, 1974), pp. 103-132.

179. Cooper, H.L.: Studies on RNA metabolism during lymphocyte activation. Transplant. Rev. 11:3-38, 1972.

180. Ranney, D.F.: An integrative approach to activation and sequential regulation of the immune response, in: Regulatory Mechanisms in Lymphocyte Activation, Proc. 11th Leukocyte Culture Conf., Tucson, Arizona, Sept. 19-23, 1976, Lucas, D.O. (Ed.), (New York: Academic Press, 1977), pp. 3-34.

181. Greaves, M. and Janossy, G.: Elicitation of selective T and B lymphocyte responses by cell surface binding ligands. Transplant. Rev. 11:87-130, 1972.

182. Schreiner, G.F. and Unanue, E.R.: Membrane and cytoplasmic changes in B lymphocytes induced by ligand-surface immunoglobulin interaction. Advan. Immunol. 24:37-165, 1976.

183. Loor, F.: Plasma membrane and cell cortex interactions in lymphocyte functions. Advan. Immunol. 30:1-120, 1980.

184. Montecucco, C., Rink, T.J., Pozzan, T. and Metcalfe, J.C.: Triggering of lymphocyte capping appears not to require changes in potential or ion fluxes across the plasma membrane. Biochim. Biophys. Acta 595:65-70, 1980.

185. Morrison, D.C. and Rudbach, J.A.: Endotoxin-cell membrane interactions leading to transmembrane signaling. Contemp. Top. Molec. Immunol. 8:187-218, 1981.

186. Rosenspire, A.J., Rosen, R. and Jacobs, D.M.: A general interactive model for B cell activation. I. The theory. Cell Biophys. 3:71-87, 1981.

187. Rosenspire, A.J. and Jacobs, D.M.: A general interactive model for B cell activation. II. Experimental verification. Cell. Biophys. 3:89-104, 1981.

188. Bloom, F.E.: General discussion and conference summary, conference on growth regulation by ion fluxes. Ann. N.Y. Acad. Sci. 339:311-325, 1980.

189. Negendank, W.G. and Collier, C.R.: Ion contents of human lymphocytes: The effects of Concanavalin A and Ouabain. Exptl. Cell Res. 101:31-40, 1976.

190. Whitney, R.B. and Sutherland, R.M.: Requirement for calcium ions in lymphocyte transformation stimulated by phytohemagglutin. J. Cell. Physiol. 80:329-338, 1972.

191. Brennan, J.K. and Lichtman, M.A.: The effect of extracellular calcium and magnesium on the proliferation of murine lymphoblasts. J. Cell. Physiol. 82:101-112, 1973.

192. Greene, W.C., Parker, C.M. and Parker, C.W.: Calcium and lymphocyte activation. Cell. Immunol. 25:74-89, 1976.

193. Whitney, R.B. and Sutherland, R.M.: Characteristics of calcium accumulation by lymphocytes and alterations in the process induced by phytohemagglutinin. J. Cell. Physiol. 82:9-19, 1973.

194. Whitfield, J.F., MacManus, J.P., Boynton, A.L., Grillan, D.J. and Isaacs, R.J.: Concanavalin A and the initiation of thymic lymphoblast DNA synthesis and proliferation by a calcium-dependent increase in cyclic GMP level. J. Cell. Physiol. 84:445-458, 1974.

195. Freedman, M.H. and Raff, M.C.: Induction of increased calcium uptake in mouse T lymphocytes by concanavalin A and its modulation by cyclic nucleotides. Nature 255:378-382, 1975.

196. Freedman, M.H.: Early biochemical events in lymphocyte activation; I. Investigations on the nature and significance of early calcium fluxes observed in mitogen-induced T and B lymphocytes. Cell. Immunol. 44:290-313, 1979.

197. Bauer, H.C., Speth, V. and Brunner, G.: Osmotic effects on the plasma membrane of thymocytes. Biochim. Biophys. Acta 507:408-418, 1978.

198. Buckhold Shank, B. and Smith, N.E.: Regulation of Cellular growth by sodium pump activity. J. Cell. Physiol. 87:377-388, 1976.

199. Buckhold, B., Adams, R.B. and Gregg, E.C.: Osmotic adaption of mouse lymphoblasts. Biochim. Biophys. Acta 102:600-608, 1965.

200. Rosenberg, H.M., Buckhold Shank, B. and Gregg, E.C.: Volume changes of mammalian cells subjected to hypotonic shock in vitro: Evidence for the requirement of a sodium pump for the shrinking phase. J. Cell. Physiol. 80:23-32, 1972.

201. Thomson, A.E.R., Bull, J.M. and Robinson, M.A.: A procedure for separating viable lymphocytes from human blood and some studies on their susceptibility of hypotonic shocks. Brit. J. Haematol. 12:433-446, 1966.

202. Casarett, A.P.: Radiation Biology, (Englewood Cliffs, NJ: Prentice-Hall, 1968).

203. Stefani, S. and Schrek, R.: Cytotoxic effects of 2 and 5 roentgens on human lymphocytes irradiated in vitro. Radiat. Res. 22:126-129, 1964.

204. Birkeland, S.A.: In vitro radiosensitivity of human T and B lymphocytes evaluated using lymphocyte transformation and rosette formation tests. Int. Archs. Allergy Appl. Immunol. 57:425-434, 1978.

205. Castellani, A., Catena, C. and Biondi, G.: Radiation sensitivity of stimulated human lymphocytes: Relation to DNA repair and cell membrane activation processes, in: Lymphocyte Stimulation; Differential Sensitivity to Radiation, Biochemical and Immunological Processes, Castellani, A. (Ed.), (New York: Plenum Press, 1978), pp. 85-96.

206. Sato, C., Kajima, K. and Matsuzawa, T.: Change in the electrophoretic behavior of X-irradiated lymphocytes by phytohemagglutinin. Int. J. Radiat. Biol. 20:97-99, 1971.

207. Myers, D.K., DeWolfe, D.E., Araki, K. and Arkinstall, W.W.: Loss of soluble materials from irradiated thymocytes in vitro. Canad. J. Biochem. Physiol. 41:1181-1199, 1963.

208. Myers, D.K.: Prevention of pycnosis in rat thymocytes. Exptl. Cell. Res. 38:354-365, 1965.

209. Chapman, I.V.: The influence of X-irradiation in vitro on the influx and efflux of potassium in thymocytes. Int. J. Radiat. Biol. 20:186, 1971.

210. Chapman, I.V. and Sturrock, M.G.: The effect of X-irradiation in vitro on the transport of potassium ions in thymocytes. Int. J. Radiat. Biol. 22:1-9, 1972.

211. Schmidtke, J.R., Ferguson, R.M. and Simmons, R.L.: Effect of x-irradiation and neuraminidase on the capacity of human lymphocytes to form rosettes with sheep red blood cells. Transplantation 22:635-638, 1976.

212. Herva, E. and Kiviniitty, K.: The effect of in vitro irradiation on the responses of human lymphocytes to PHA, PPD, and allogenic cells. Strahlentherapic 149:504-512, 1975.

213. Rickinson, A.B. and Ilbery, P.L.T.: The effect of radiation upon lymphocyte response to PHA. Cell Tissue Kinet. 4:549-562, 1971.

214. Sprent, J., Anderson, R.E. and Miller, J.F.A.P.: Radiosensitivity of T and B lymphocytes. II. Effect of irradiation on response of T cells in alloantigens. Eur. J. Immunol. 4:204-210, 1974.

215. Anderson, R.E., Sprent, J. and Miller, J.F.A.P.: Radiosensitivity of T and B lymphocytes. I. Effect of irradiation on cell migration. Eur. J. Immunol. 4:199-203, 1974.

216. Baranski, S.: Effect of chronic microwave irradiation on the blood forming system of guinea pigs and rabbits. Aerospace Med. 42:1196-1199, 1971.

217. Baranski, S.: Effect of microwaves on the reactions of the white blood cells system. Acta Physiol. Polonica 23:685-695, 1972.

218. Czerski, P.: Microwave effects on the blood-forming system with particular reference to the lymphocyte. Ann. N.Y. Acad. Sci. 247:232-241, 1975.

219. Huang, A.T., Engle, M.E., Elder, J.A., Kinn, J.B., Ward, T.R.: The effects of microwave radiation (2450 MHz) on the morphology and chromosomes of lymphocytes. Radio. Sci. 12(6S):173-177, 1977.

220. Prince, J.E., Mori, T.H., Frazier, J.W. and Mitchell, J.C.: Cytological aspect of RF radiation in the monkey. Aerospace Med. 43:759-761, 1972.

221. Liddle, C.G., Putnam, J.P., Ali, J.S., Lewis, J.Y., Bell, B., West, M.W. and Lewter, O.H.: Alteration of circulating antibody response of mice exposed to 9-Ghz pulsed microwaves. Bioelectromagnetics 1:397-404, 1980.

222. Wiktor-Jedrejczak, W., Ahmed, A., Czerski, P., Leach, W.M., and Sell, K.W.: Immune response of mice to 2450-MHz microwave radiation: Overview of immunology and empirical studies of lymphoid splenic cells. Radio. Sci. 12(65):209-219, 1977.

223. Stodolnik-Baranska, W.: Lymphoblastoid transformation of lymphocytes in vitro after microwave irradiation. Nature 214:102-103, 1967.

224. Stodolnik-Baranska, W.: The effects of microwaves on human lymphocyte cultures, in: Biological Effects and Health Hazards of Microwave Radiation, Czerski, P. Ostrowski, K., Shore, M.L., Silverman, C., Suess, M.J. and Waldeskog, B., (Warsaw, Poland: Polish Medical Publishers, 1974), pp. 189-195.

225. Wiktor-Jedrezejezak, W., Ahmed, A., Czerski, P., Leach, W.M.: Microwaves induce an increase in the frequency of complement receptor-bearing lymphoid spleen cells in mice. J. Immunol. 118:1499-1502, 1977.

226. Wiktor-Jedrzejczak, W., Ahmed, A., Czerski, P., Leach, W.M., and Sell, K.W.: Increase in the frequency of Fc receptor (FcR)-bearing cells in the mouse spleen following a single exposure of mice to 2450-MHz microwaves. Biomedicine 27:250-252, 1977.

227. Sulck, K., Schlagel, C.J., Ho, H.S., Leach, W.M., Ahmed, A., Woody, J.N. and Wiktor-Jedrzejczak, W.: Biologic effect of microwave exposure. I. Threshold energy requirement for the induction of an increase in complement receptor positive (CR^+) mouse spleen cells following exposure to 2450 MHz microwaves. Radiat. Res. 83:127-137, 1980.

228. Schlagel, C.J., Sulek, K., Ho, H.S., Leach, W.M., Ahmed, A. and Woody, J.N.: Biological effects of microwave exposure. II. Studies on the mechanisms controlling susceptibility to microwave-induced increases in complement receptor-positive spleen cells. Bioelectromagnetics 1:405-414, 1980.

229. Wiktor-Jedrzejezak, W., Ahmed, A., Czerski, P., Leach, W.M., and Sell, K.W.: The effect of microwaves (2450 MHz) on the immune system in mice: Cell metabolism studies using labelled nuclei acids and protein substrates Bioelectromagnetics 1:161-170, 1980.

230. Schlagel, C.J. and Wiktor-Jedrzejczak, W.: Possible humoral mechanism of 2450 MHz microwave-induced increase in complement receptor positive cells as studied using a modified diffusion chamber culture technique. Bioelectromagnetics 1:247, 1981.

231. Smialowiez, R.J., Kinn, J.B. and Elder, J.A.: Perinated exposure of rats to 2450-MHz CW microwave radiation: Effects on lymphocytes. Radio. Sci. 14(6S):147-153, 1979.

232. Smialowicz, R.J.: The effect of microwaves (2450 MHz) on lymphocyte blast transformation in vitro, in: Biological Effects of Electromagnetic Waves, Proc. USNC/URSI Ann. Meeting, Boulder, Colorado, October 20-23, 1975, eds. C.C. Johnson and M.L. Shore (Rockville, MD: Bureau of Radiological Health, 1976) HEW Publication (FDA) 77-8010.

233. Hakim, A.A.: Thermal injury: relase of a cytotoxic factor. Experientra 15:865-867, 1973.

234. Miller, C.L. and Claudy, B.J.: Suppressor T-cell activity induced as a result of thermal injury. Cell. Immunol. 44:201-208, 1979.

235. Harris, J.W.: Effects of tumor-like assay conditions, ionizing radiation, and hyperthermia on immune lysis of tumor cells by cytotoxic T-lymphocytes. Cancer Res. 36:2733-2739, 1976.

236. MacDonald, H.R.: Effect of hyperthermia on the functional activity of cytotoxic T-lymphocytes. J. Natl. Cancer Inst. 59:1263-1268, 1977.

237. Harris, J.W. and Meneses, J.J.: Effects of hyperthermia on the production and activity of primary and secondary cytolytic T-lymphocytes in vitro. Cancer Res. 38:1120-1126, 1978.

238. Schrek, R.: In vitro methods for measuring viability and vitality of lymphocytes exposed to 45°, 47°, and 50°C. Cryobiol. 2:122-128, 1965.

239. Schrek, R.: Sensitivity of normal and leukemic lymphocytes and leukemic myeloblasts to heat. J. Natl. Cancer Inst. 37:649-654, 1966.

240. Schrek, R., Chandra, S., Molnar, Z. and Stefani, S.S.: Two types of interphase death of lymphocytes exposed to temperatures of 37-45°C. Radiat. Res. 82:162-170, 1980.

241. Chapman, I.V., MacNally, N.A. and Tucker, S.: Ultrasound-induced changes in rates of influx and efflux of potassium ions in rat thymocytes in vitro. Ultrasound Med. Biol. 6:47-58, 1979.

242. Shortman, K.: Separation methods for lymphocyte populations. Contemp. Top. Molec. Immunol. 3:161-203, 1974.

243. Williams, N., Kraft, N. and Shortman, K.: The separation of different cell classes from lymphoid organs. VI. The effect of osmolarity of gradient media on the density distribution of cells. Immunology 22:885-899, 1972.

244. Shortman, K., Williams, N. and Adams, P.: The separation of different cell classes from lymphoid organs. V. Simple procedures for the removal of cell debris, damaged cells and erthroid cells from lymphod cell suspensions. J. Immunol. Methods 1:273-287, 1972.

245. Von Boehmer, H. and Shortman, K.: The separation of different cell classes from lymphoid organs. IX. A simple and rapid method for removal of damaged cells from lymphoid cell suspensions. J. Immunol. Methods 2:293-301, 1973.

246. Andreasen, P.A., Schaumburg, B.P., Østerlind, K., Vinten, J., Gammeltoft, S. and Gliemann, J.: A rapid technique for separation of thymocytes from suspensions by centrifugation through silicon oil. Anal. Biochem. 59:610-616, 1974.

247. Gelsthorpe, K. and Doughty, R.W.: An easily produced micro-cytotoxic test plate with a further modification of the standard micro-cytotoxic technique for lymphocyte typing. Med. Lab. Technol. 28:22-25, 1971.

248. Brown, J.: A micro-method for the study of in vitro lymphocyte transformation to specific antigens. J. Immunol. Methods 18:17-32, 1977.

249. Oppenheim, J.J. and Rosenstreich, D.L.: Lymphocyte transformation: Utilization of automatic harvesters, in: In Vitro Methods in Cell Mediated and Tumor Immunity, Bloom, B.R. and Glade, P. (Eds.), (New York: Academic Press, 1971), pp. 573.

250. Oppenheim, J.J. and Schecter, B.: Lymphocyte transformation, in: Manual of Clinical Immunology, Rose, N.R. and Friedman, H. (Eds.), (Washington, DC: American Society of Microbiology, 1976), pp. 81-94.

251. Strong, D.M., Ahmed, A.A., Thurman, G.B. and Sell, K.W.: In vitro stimulation of murine spleen cells using a microculture system and a multiple automated sample harvester. J. Immunol. Methods 2:279-291, 1973.

252. Waithe, W.I. and Hirschhorn, K.: Lymphocyte response to activators, in: Handbook of Experimental Immunology, Vol. 2, Cellular Immunology, 3rd ed., Weir, D.M. (Ed.), Oxford: Blackwell Scientific Publications, 1978), pp. 26.1-26.10.

253. Dei, R. and Urbano, P.: Biometrical implications of factorial experiments for the study of lymphocyte mitogenic response. J. Immunol. Methods 15:169-181, 1977.

254. Angus, K. and Yang, T.J.: Lymphocyte response to phytohemagglutinin: Temporal variation in normal dogs. J. Immunol. Methods 21:261-269, 1978.

255. Bliss, C.I., Cochran, W.G. and Tukey, J.W.: A rejection criterion based upon the range. Biometrika 43:418-422, 1956.

256. Dixon, W.J.: Processing data for outliers. Biometrics 9:74-89, 1953.

257. Schwan, H.P.: Field interaction with biological matter. Ann. NY Acad Sci. 303:198-213, 1977.

258. Lorrain, P. and Corson, D.R.: Electromagnetic fields and waves, 2nd ed. (San Francisco: W. H. Freeman, 1970).

259. Mild, K.H., Lövdahl, L., Lövstrand, K-G. and Lovtrup, S.: Effect of high-voltage pulses on the viability of human leukocytes in vitro. (Submitted for publication).

260. Cope, F.W.: A primer of water structuring and cation association in cells, I. Introduction: The big picture. Physiol. Chem. Phys. 8:479-483, 1976.

261. Cope, F.W.: A primer of water structuring and cation association in cells, II. Historical notes, present status, and future directions. Physiol. Chem. Phys. 8:569-574, 1976.

262. Cope, F.W.: Pathology of structured water and associated cations in cells (The tissue damage syndrome) and its medical treatment. Physiol. Chem. Phys. 9:547-553, 1977.

263. Whitfield, J.F., Brohee, H. and Youdale, T.: The effects of x-radiation on the chromatin structure and histone components of rat thymocyte nuclei. Exptl. Cell. Res. 36:341-353, 1964.

264. Apelgot, S., David, R., Rebeyratte, N. and Cantrel, C. Concerning DNA liquid scintillation measurement of ^{14}C and ^{3}H radioactivity, in: Liquid Scintillation Counting; Recent Applications and Development, II. Sample Preparation and Applications, Peng C.-T., Horrocks, D.L. and Alpen, E.L. (Eds.) (New York: Academic Press, 1980), pp. 53-67.

265. Coutinho, A., Möller, G., Anderson, J. and Bullock, W.W.: In vitro activation of mouse lymphocytes in a serum-free medium: Effect of T and B cell mitogens on proliferation and antibody synthesis. Eur. J. Immunol. 3:299-306, 1973.

266. SAS User's Guide 1979 Edition, Helwig, J.J. and Council, K.A. (Eds.), Raleigh, NC: SAS Institute, Inc., 1979), pp. 238-239.