The effect of calcium-tuned cyclotron resonance upon the proliferation rate of adult T-cell leukemia cells

by Scott Patrick Simon

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Electrical Engineering
Montana State University

Abstract:
This thesis describes a Master's project which consisted of the preliminary stages of a larger project which will be extended by others over the next year. The purpose of the project was to develop and apply procedures to test the effect of electric and magnetic fields, precisely adjusted to cyclotron resonance for the calcium ion, upon the growth rate of Adult T-Cell Leukemia (ATL) cells. First a procedure was developed to test the response ATL cell growth rate to varying levels of extracellular calcium. Then a procedure was designed and developed to test for the effects of magnetic fields, precisely tuned to the cyclotron resonance of calcium ion, upon the growth rate of the cells. In the first procedure, after incubation in low-calcium medium with varying levels of added calcium, cells grown in each of six different concentrations were counted and compared. Proliferation increased with increasing calcium up to a peak value, then began to diminish with increasing calcium. In the second procedure, cells were again grown in medium with six different levels of added calcium. Experimental cells (grown while exposed to cyclotron resonance) were counted and compared to counts of control cells (grown under identical conditions except not exposed to resonance). The experimental cells grew faster than controls in medium with lower levels of calcium and reached a peak at a lower level of added calcium than did the controls. These results suggest the possibility that cyclotron resonance increases the amount of calcium apparent to the cell, either by increasing the flow of calcium ions into the cell or by increasing the activity of calcium ion originally in the cell. These data also indicate that further study should be conducted in order to ascertain the response of these cells to tuning to other ions, and also to precisely localize the site of interaction between the cells, the ions, and the magnetic fields.

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Scott Patrick Simon

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Scott Patrick Simon

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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Signed Scott P. Simon

Date 12-5-89

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ABSTRACT

This thesis describes a Master's project which consisted of the preliminary stages of a larger project which will be extended by others over the next year. The purpose of the project was to develop and apply procedures to test the effect of electric and magnetic fields, precisely adjusted to cyclotron resonance for the calcium ion, upon the growth rate of Adult T-Cell Leukemia (ATL) cells. First a procedure was developed to test the response ATL cell growth rate to varying levels of extracellular calcium. Then a procedure was designed and developed to test for the effects of magnetic fields, precisely tuned to the cyclotron resonance of calcium ion, upon the growth rate of the cells. In the first procedure, after incubation in low-calcium medium with varying levels of added calcium, cells grown in each of six different concentrations were counted and compared. Proliferation increased with increasing calcium up to a peak value, then began to diminish with increasing calcium. In the second procedure, cells were again grown in medium with six different levels of added calcium. Experimental cells (grown while exposed to cyclotron resonance) were counted and compared to counts of control cells (grown under identical conditions except not exposed to resonance). The experimental cells grew faster than controls in medium with lower levels of calcium and reached a peak at a lower level of added calcium than did the controls. These results suggest the possibility that cyclotron resonance increases the amount of calcium apparent to the cell, either by increasing the flow of calcium ions into the cell or by increasing the activity of calcium ion originally in the cell. These data also indicate that further study should be conducted in order to ascertain the response of these cells to tuning to other ions, and also to precisely localize the site of interaction between the cells, the ions, and the magnetic fields.

STATEMENT OF HYPOTHESIS

The objective of this project was to develop and perform procedures to test the following hypothesis: "Can cyclotron resonance, tuned to frequencies corresponding to the charge to mass ratio of physiologically important ions, affect the growth rate of transformed cells?" The work consisted of designing an experimental procedure aimed at determining whether or not the effects of resonance upon several different types of cells shown by previous work can be generalized to transformed cells.

THEORY

Cyclotron resonance is a physical phenomenon which involves interactions between a magnetic field (B-field), an electric field (E-field), and one or more charged particles. The fields act as sources of field energy which is coupled into the particles in the form of kinetic energy. Experiments by Blackman et al. (1) showed that a set of response frequencies given by

$$w_p = K(2n+1)B_o \qquad [1]$$

with w_p = the radian frequency of the applied time-varying electric field

K = a proportionality constant

n = 0,1,2,3,...

B_o = the magnitude of the constant, DC magnetic flux density

were effective in increasing the flow of calcium ions out of chick brain tissue. Liboff (2) observed that the calcium flow response appeared to occur at the cyclotron resonance frequency of some biologically important ions. As a result of that observation, equation [1] was rewritten in a more useful form as

$$w_p = (q/m)(2n+1)B_o \qquad [2]$$

where K has been replaced by q/m, the charge-to-mass ratio of the charged particle (biologically important ion). The

Blackman study (1) also showed that the response of the brain tissue was different for values of B that were one-third or two-thirds that of B_o. For $B = B_o/3$ and $B = B_o$, the tissue showed increased calcium flow, but there was no response when $B = 2B_o/3$. This suggests that the tissue was responding to harmonics of a fundamental frequency. We will see below that cyclotron resonance theory predicts a response at odd harmonics of w_p and no response at even harmonics.

This paper will first consider a simplified, intuitive model of a single charged particle (in vacuum) being exposed to a single axis DC magnetic field. Next, a sinusoidally time varying E-field will be added to the model. Finally, the mathematical description of the model is developed.

A Charged Particle in a Single-Axis DC Magnetic Field

Assumptions:

(1) Free space

  (a) μ and ϵ are not functions of the corresponding field quantities

  (b) the particle experiences no resistive forces (losses)

(2) The magnetic field has only a single vector component ($\mathbf{B} = B_z\mathbf{z}$)

(3) The magnetic field is constant in time

(4) The magnitude of the electric field is zero

Consider a DC magnetic field oriented along the z-axis (**B**

= $B_z\mathbf{z}$) and a particle with mass m and electrical charge q having initial position (x, y, z,) = (x_o, 0, 0) and initial velocity (V_x, V_y, V_z) = (0, V_o, 0). This situation is pictured in Figure 1.

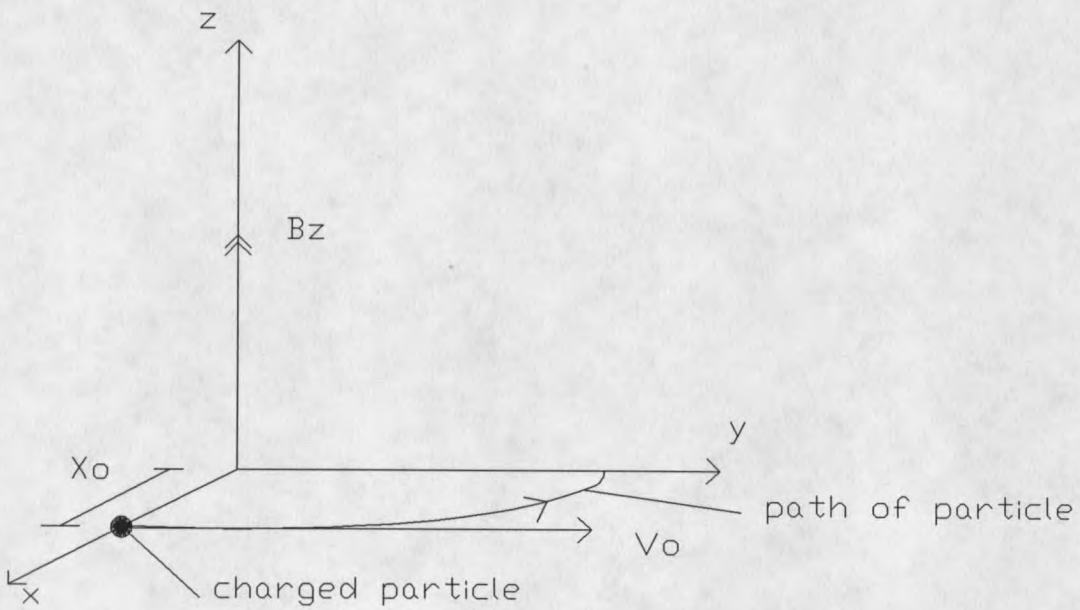


Figure 1. Charged particle in a single-axis DC magnetic field.

The magnetic force acting on the particle is given by

$$\mathbf{F} = m\mathbf{a} = md\mathbf{V}/dt = q\mathbf{E} + q(\mathbf{V}x\mathbf{B}) \qquad [3]$$

As a result of assumptions 2 and 4, equation [3] reduces to

$$\mathbf{F} = m(d\mathbf{v}/dt) = q(0) + q[\mathbf{V}x(0\mathbf{x} + 0\mathbf{y} + B_z\mathbf{z})] \qquad [4]$$

Equation [4] states that the force on the particle will have a magnitude of qV_0B_z. Because of the vector cross product, the force will always be perpendicular to the particle velocity. It follows that the particle will travel in a circular path (shown as a dotted line in Figure 1), moving at a constant speed ($|\mathbf{V}|$). (3) The kinetic energy, given by

$$KE = (1/2)\ m|\mathbf{V}|^2 \qquad [5]$$

is also constant. The field adds no energy to the particle; it merely provides a force which constrains the particle to a circular orbit about the z-axis. (4) The radius of the circular path will be inversely proportional to B_z. (3) The radian frequency of revolution is called the cyclotron frequency and is given by

$$w_{ion} = B_z(q/m)\ . \qquad [6]$$

Addition of an AC Electric Field

If we assume all of the applied electromagnetic fields to be sinusoidal (or cosinusoidal), we can use a convenient form of the time varying electric field, representing $\mathbf{E}$ as

$$\mathbf{E} = Re[E_o(e^{jwt})] = E_o\cos wt\ . \qquad [7]$$

If all the time varying fields have this time form it is convenient to then drop the "Re" and suppress the e^{jwt} term. It is then recognized that any time derivative will simply be

$$\delta^n\mathbf{E}/\delta t^n = (jw)^nE_o\ . \qquad [8]$$

As an example

$$\delta^2\mathbf{E}/\delta t^2 = (jw)^2E_o = -w^2 \ . \qquad [9]$$

where E_o = a general function of space.

Now suppose that the particle is in steady-state, moving in a circle around the z-axis (shown in Figure 2 as viewed from the negative z direction).

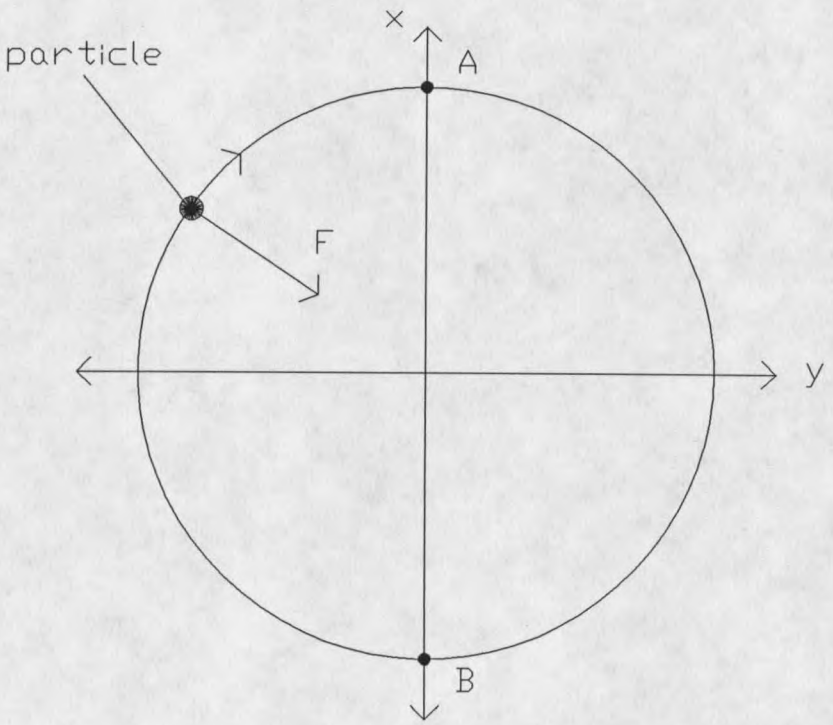


Figure 2. Steady-state situation of a charged particle in a DC magnetic field.

Consider the application of an E-field in the x-y plane, given in cylindrical coordinates by

$$\mathbf{E} = (E_o\cos wt)\phi \qquad [10]$$

where ϕ is a unit vector pointing in the direction of increasing angle ϕ (i.e. counterclockwise when the observer looks down the z axis). The position of the positive maximum

of the E-field at three different times is shown in Figure 3, again as viewed from the negative z direction. The particle is now acted on by a force given by

$$\mathbf{F} = q\mathbf{E} + q(\mathbf{V}\times\mathbf{B}) \quad . \qquad [11]$$

It is seen from comparison of Figures 2 and 3 that if $w=w_o$, the maximum of the $q\mathbf{E}$ component of force on the particle acts in the direction of particle velocity through the entire revolution of the particle about the z-axis. Energy is constantly being coupled into the particle by the E-field, increasing the kinetic energy of the particle. (3)

If w is slightly greater than w_p, the $q\mathbf{E}$ force will first act in the direction of particle velocity. However, since the position of the electric field is moving faster than the particle, the position of the negative half cycle of the $q\mathbf{E}$ force (acting opposite to the direction of particle velocity), will soon catch up to the particle. For the time that the negative force acts on it, energy is drained from the particle. Thus, for the case of w slightly greater than w_p, a particle is alternately pushed and pulled by the electrical force as it orbits. By similar reasoning, if w is slightly less than w_p the particle will also experience a force alternating between positive (push) and negative (pull). In either case energy is not constantly coupled into the particle. (3)

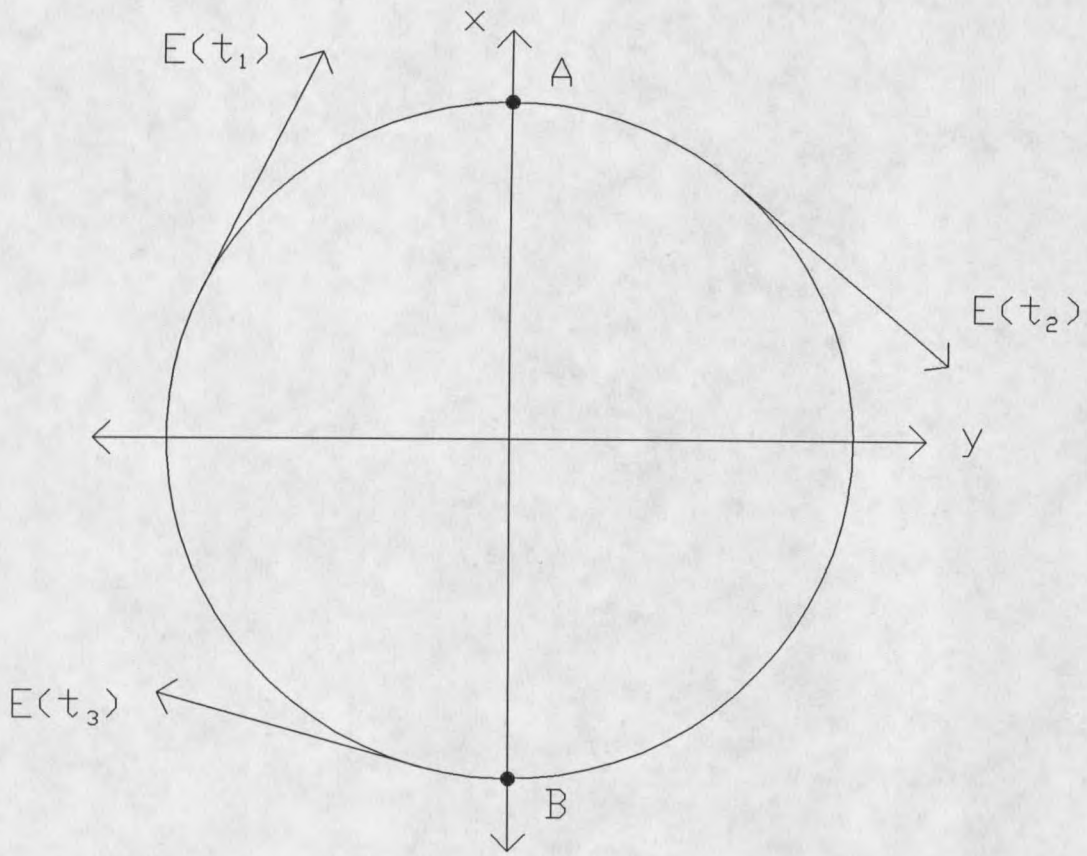


Figure 3. Position of the electric field at three different times.

Experiments by McLeod et al. (5) have shown that the E-field will transfer net energy to the particle if w equals an odd harmonic of w_p. This may be understood by examining Figures 4 and 5.

Consider Figure 4, which shows both the cyclotron frequency and its second harmonic ($2w_p$). If we again assume that $w_{ion}=w_p$, the waveform of the fundamental in Figure 4 corresponds to the path of the particle in Figure 2. Thus, one positive half cycle of the fundamental in Figure 4 corresponds to the particle moving around the half circle

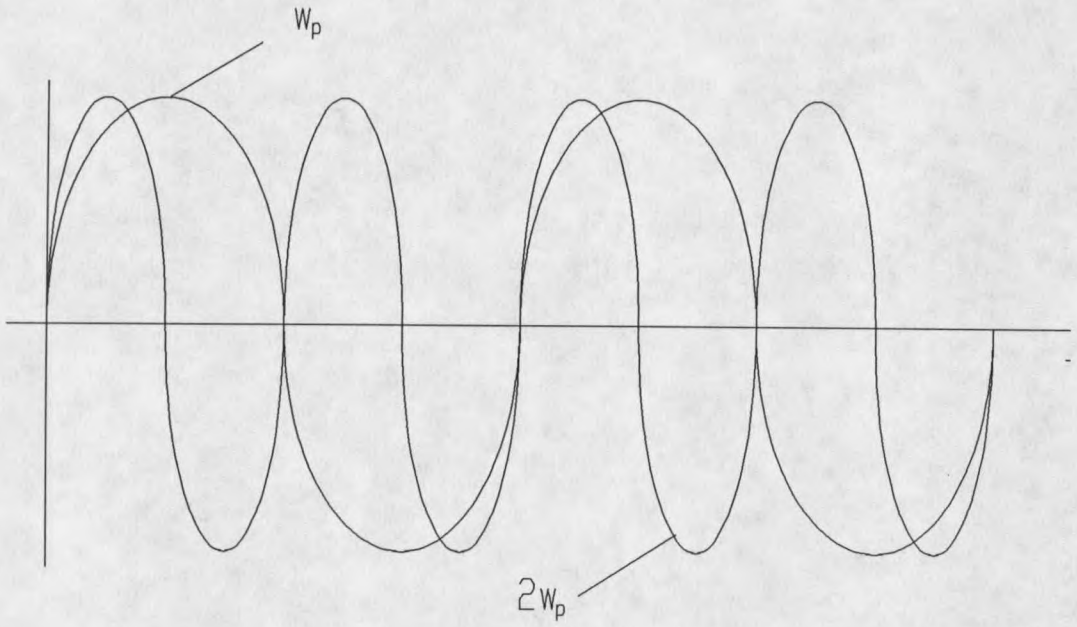


Figure 4. Cyclotron frequency (w_p) and second harmonic ($2w_p$).

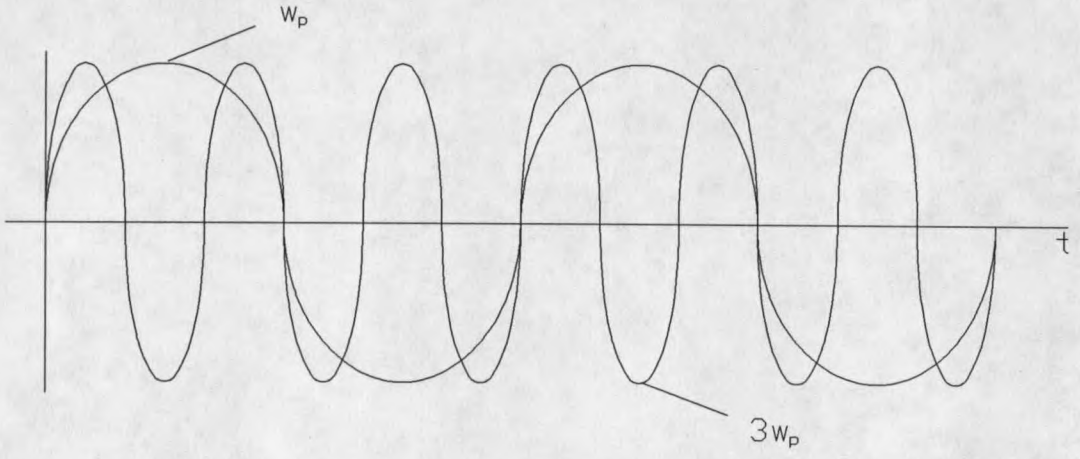


Figure 5. Cyclotron frequency (w_p) and third harmonic ($3w_p$).

from point A to point B in Figure 2 and the negative half cycle represents the path from B to A. The wave form of the second harmonic represents the qE component of the force.

We see in Figure 4 that for an electric field with a frequency of $2w_p$ (or any even harmonic), the force will act positively on the particle over one quarter of a revolution and negatively over the next. Thus, for any full revolution of the particle, the average time during which the qE force pushes the particle along its path equals the time during which the qE force opposes the motion of the particle. No net energy from the E-field is coupled into the particle.

Figure 5 shows the fundamental (particle path) and the third harmonic (qE) force. We see here that with $w = 3w_p$ (or any odd harmonic), the force pushes the particle along its path for two thirds of every half revolution. In this case, for every full revolution the time during which the E-field is pushing on the particle is greater than the time during which it is impeding particle motion. Thus, for any odd harmonic, some net energy is coupled from the E-field into the particle. This idealized model predicts that eventually an infinite amount of energy could be coupled into the particle. In reality, however, the particles are not in vacuum.

The real case, involves damping resulting from collisions between the particle in question and other particles. Adding a damping term to equation [3] yields

$$\mathbf{F} = q\mathbf{E} + q(\mathbf{V} \times \mathbf{B}) - m\mathbf{V}/\tau \qquad [12]$$

where

M = the ion mass

V = the ion velocity vector

τ = the mean time between collisions of the ion and other particles.

This new term accounts for a velocity-dependent energy limiting mechanism which prevents the particle from attaining infinite kinetic energy. (3)

The loss term represents one of the difficulties in applying cyclotron resonance theory to a biological system. If it is possible for cyclotron resonance to affect the rate of ion movement across the cell membrane, the interaction must occur either outside the cell membrane or within the ion channel of the membrane. The extracellular environment contains a large number of particles with which an ion might collide. The typical density of those particles suggests a collision time τ which would be so short that it is unlikely an ion would ever undergo even a single circular orbit as pictured in Figure 2. However, the current thinking about membranes and ion transport across those membranes suggests that the density of particles inside the membrane channel is much lower than outside of the cell. In fact, it is possible that as few as one or two ions occupy the channel at any one instant. (3) This implies that if cyclotron resonance theory is to explain the interaction between low level electric and

magnetic fields, physiologically important ions, and biological systems, it must to applied to the environment inside the membrane ion channel.

If the assumption is made that the interaction takes place inside the channel, additional constraints are placed on the motion of the ion as it passes through. The radius of the orbit shown in Figure 2 must now be less than or equal to the radius of the membrane channel minus the radius of the ion. Thus, a viable explanation must consider the ion to be constrained rather than free. Classical membrane channel theory assumes the path of the ion through the channel to be linear or at least "statistically" linear. (3) Since cyclotron resonance concerns particles in circular motion, the motion of a particle as it passes through the channel under the influence of the fields is probably circular or helical.

The next section develops a preliminary mathematical model corresponding to the previous physical description of the simplistic case of a single free particle with both electric and magnetic fields applied. The model takes into consideration a loss term, but no channel walls. In order to produce a viable theory of the interaction of fields with biological systems, this model will eventually need to be expanded to apply to a constrained particle.

Mathematical Model Corresponding to the Physical Model

Using equation [2a] and an expression for current density (**J**), the mathematical description of the model described may be derived. The starting point of this development is equation [3] rewritten as

$$\mathbf{F} = mjw\mathbf{V} = q(\mathbf{V} \times \mathbf{B}') + q\mathbf{E} - mV/\tau. \qquad [13]$$

The following assumptions are made:

(1) The time variation is of the form $f(t) = e^{jwt}$ so that $Re[\, f(t)] = Re\, [e^{jwt}] = \cos wt$. $B' = B_x\mathbf{x} + 0y + B_z\mathbf{z}$.

(2) The plasma density is low enough so that interparticle forces can be ignored.

(3) Let **B** have two components, B_x and B_z (i.e. $B_y = 0$).

(4) The sign of the ion charge q is taken as positive for simplicity. It is easily changed to negative in the final result for anions.

Let us write equation [13] in component form:

$$mjwV_x = qE_x + qV_yB_z - mV_x/\tau \qquad [14]$$

$$mjwV_y = qE_y + qV_zB_x - qV_xB_z - mV_y/\tau \qquad [15]$$

$$mjwV_z = qE_z - qV_yB_x - mV_z/\tau \qquad [16]$$

Equations [14-16] may be manipulated to yield expressions (not included here) for V_x, V_y, and V_z in terms of **B** and **E**. Those expressions are then substituted into

$$\mathbf{J} = Nq\mathbf{V} = \sigma\mathbf{E} \qquad [17]$$

where

J = the current density vector (amp/m^2)

N = the density of charged particles in ($\#/m^3$)

σ = the conductivity tensor in $(w\text{-}m)^{-1}$

to solve for σ. The general form of σ is

$$\sigma = \begin{bmatrix} \sigma_{11} & \sigma_{12} & \sigma_{13} \\ \sigma_{21} & \sigma_{22} & \sigma_{23} \\ \sigma_{31} & \sigma_{32} & \sigma_{33} \end{bmatrix} \qquad [18]$$

Substituting the expressions for V_x, V_y, and V_z into equation [17] and performing some algebra yields

$$\sigma_{11} = (Nq^2\tau/mK)\ [(w_{Bx}{}^2\tau^2 + A^2)/A] \qquad [19]$$

$$\sigma_{22} = (Nq^2\tau/mK)\ (A) \qquad [20]$$

$$\sigma_{33} = (Nq^2\tau/mK)\ [(w_{Bz}{}^2\tau^2 + A^2)/A] \qquad [21]$$

$$\sigma_{12} = -\sigma_{21} = (Nq^2\tau/mK)\ (w_{Bz}\tau) \qquad [22]$$

$$\sigma_{13} = -\sigma_{31} = (Nq^2\tau/mK)\ (w_{Bz}w_{Bx}\tau^2) \qquad [23]$$

$$\sigma_{23} = -\sigma_{32} = (Nq^2\tau/mK)\ (w_{Bx}\tau) \qquad [24]$$

where $A = (jw\tau + 1)$ [25]

$$K = (w_{Bz}{}^2 + w_{Bx}{}^2)\tau^2 + A^2 \qquad [26]$$

$w_{Bx} = qB_x/m$ = resonant frequency for B_x [27]

$w_{Bz} = qB_z/m$ = resonant frequency for B_z [28]

Note that each term in the σ tensor is multiplied by the same term, $(Nq^2\tau/mK)$. If equation [26] is rewritten as

$$K = 1 + [(w_{Bz}{}^2 + w_{Bx}{}^2 - w^2)]\tau^2 + 2jw\tau\ , \qquad [29]$$

we see that as the radian frequency w approaches $(w_{Bz}{}^2 + w_{Bx}{}^2)$, the real part of the denominator in each term of the σ tensor approaches a minimum, i.e. each element in the tensor approaches a maximum. This suggests that

$$w = (w_{Bz}^2 + w_{Bx}^2)^{1/2} \qquad [30]$$

is a critical frequency for the ions in the plasma being considered. (3) Due to the complexity of the expressions it is not yet clear exactly what occurs at this "resonance point." Let us consider the less general case where

$$\mathbf{B} = B_o\mathbf{z} \qquad [31]$$

and

$$\mathbf{E} = E_x\mathbf{x} + E_y\mathbf{y}, \qquad [32]$$

i.e. the magnetic field has a single component along the z axis and the electric field is entirely perpendicular to **B**. The conductivity tensor becomes

$$\sigma' = \sigma_o[T]/[1 + (w_{Bz}^2 - w^2)\tau^2 + 2jw\tau] \qquad [33]$$

where

$$T = \begin{bmatrix} (1+jw\tau) & w_{Bz}\tau & 0 \\ -w_{Bz}\tau & (1+jw\tau) & 0 \\ 0 & 0 & 0 \end{bmatrix} \qquad [34]$$

and

$$\sigma_o = Nq^2\tau/m \ . \qquad [35]$$

Substituting σ' into equation [17] yields the velocity vector **V** which is used in the expression for energy

$$W = 1/2\ m\ \mathbf{V} \cdot \mathbf{V}^* \qquad [36]$$

where **V*** = the complex conjugate of **V**.

The resulting energy expression is

$$W = (E_o^2/2)(q^2\tau^2/m)[1 + (w_{Bz}^2 + w^2)\tau^2]/[D] \qquad [37]$$

where

$$E_o = E_x^2 + E_y^2 \qquad [38]$$

and

$$D = [1 + (w_{Bz}^2 - w^2)\tau^2]^2 + 4w^2\tau^2. \qquad [39]$$

Examining the energy expression as τ becomes infinite (no collisions) yields

$$W = (q^2/2m)[(w_{Bz}^2 + w^2)/(w_{Bz}^2 - w^2)^2]E_o \qquad [40]$$

Equation [40] mathematically confirms the previous intuitive statement that without an energy limiting mechanism (accounted for by the inclusion of collision term τ in equation [12]), the energy coupled from the fields into the particle would be infinite at $w = w_{Bz}$. (3)

The conductivity tensor (equation [33]) can be rewritten in a form more appropriate to an ion following a helical path. Let us define a current density for a left rotating (counterclockwise about the z axis) ion as

$$J_L = J_x - jJ_y = \sigma_L E_L \qquad [41]$$

and $$J_R = J_x + jJ_y = \sigma_L E_L \qquad [42]$$

for an ion with right rotation (clockwise about the z axis). Combining equations [33], [41] and [42] yields

$$J_L = (\sigma_{11} + j\sigma_{12})(E_x - jE_y) = \sigma_L E_L \qquad [43]$$

$$J_R = (\sigma_{11} - j\sigma_{12})(E_x + jE_y) = \sigma_L E_L \qquad [44]$$

Thus,

$$\sigma_{L,R} = \sigma_o[1 + j(w \pm w_{Bz})\tau]/[(C) + 2jw\tau] \qquad [45]$$

and

$$Re(\sigma_{L,R}) = \sigma_o[1 + j(w \pm w_{Bz})^2\tau^2]/[(C)^2 + 4w^2\tau^2 \qquad [46]$$

where $C = 1 + (w_{Bz}^2 - w^2)\tau^2$ [47]

Analyzing equations [46] and [47] reveals that $Re(\sigma_L)$ reaches a maximum when $w = w_{Bz}$. In fact, the maximum value is σ_o as defined in equation [35]. The value of $Re(\sigma_R)$ when $w=w_{Bz}$ is given by

$$Re(\sigma_R)\Big|_{w=w_{Bz}} = \sigma_o/(1 + 4w_{Bz}^2\tau^2) \quad [48]$$

This simple model suggests that if the ion moves through the membrane in a helical path, it may be possible to increase its conductivity and therefore the rate at which it moves. (3) The expression for $Re(\sigma_L)$ in equation [46] exhibits a resonance curve with a maximum value of σ_o and a width set by τ, the collision time. As yet, no direct connection has been made experimentally between a cell membrane channel and the conductivity expression. However, this simplified model represents a viable starting point for seeking a more complete understanding of the interaction between biological systems and low energy electric and magnetic fields.

The Involvement of the Calcium Ion in Biological Systems

Various types of cations (e.g. Li^{2+}, K^+, Mg^{2+}, Ca^{2+}, etc.) have been implicated in many biological functions. (6) This section will focus on the involvement of Ca^{2+} in biological processes. This involvement will first be explained in general, followed by several examples.

There are two major types of ion involvement in biological functions. The first of these is a non-regulatory role. The ion is not involved in the triggering of the function; it is merely an essential component of the function mechanism. (6) This type of ion involvement is called passive. A useful analogy is the propulsion system of an automobile. Ions in a passive role correspond to the oxygen with which gasoline reacts in the propulsion system. It is necessary to the operation of the system, but not involved in the initiation of the system response.

An example of Ca^{2+} as a passive, non-regulatory component of a function is in the formation of fibrin clots in mammals.(6) Calcium ion is essential to the activity of five enzymes which form a portion of the cascade which results in fibrin clot formation. The ion, however, is not involved as a step in the pathway between trigger mechanism and response mechanism. It merely enables a section of the chemical pathway of clot formation to operate.

The second type of ion involvement is as a regulator. The ion is directly involved in mediating the effect of the primary signal for the response. This type of ion involvement is called active. (6) In the automobile analogy, the ions in an active role correspond to the ignition wires of the system. They connect the trigger mechanism (ignition key) to the responding mechanism (engine).

Because of the enormous Ca^{2+} concentration gradient which normally exists across cell membranes, the calcium ion is uniquely situated to act as an active regulator of many different biological responses. (6) An example of this is muscle contraction in vertebrates. The function of calcium ions is not to enable the chemical reactions producing the energy for a contraction. Rather, they couple the trigger mechanism to an energy reservoir which has been charged by the metabolism of the muscle cell. Rapid muscle contraction, however, is far too rapid to be accounted for by the diffusion of Ca^{2+} down a concentration gradient. Instead, an organelle inside the cell called the sarcoplasmic reticulum (SR) is involved. (7) The SR can alternately sequester or release Ca^{2+} depending upon the immediate needs of the contraction system. During periods of muscle inactivity, the SR sequesters excess calcium from the sarcoplasm (the region inside the membrane of a muscle cell). When a nerve impulse initiates a contraction, the SR rapidly discharges Ca^{2+} into the cytoplasm. The increase in calcium concentration in the cytoplasm links the trigger (nerve impulse) to the intracellular contractile proteins (response mechanism components), which in turn contract the muscle. (7) The above examples, as well as many other types of biological phenomena are referred to as threshold phenomena.

Threshold phenomena are "cellular events involving the transition of a cell from one 'state' to another." (6)

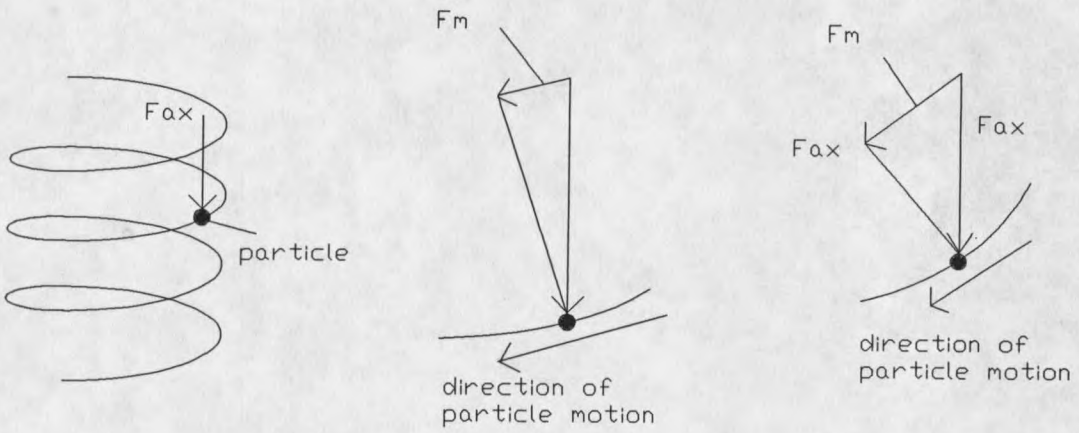
Phenomena involving transition between two "states" are termed binary responses. Examples of binary responses range from sensory responses such as nerve impulse conduction and visual pathways to cell and tissue development responses such as cell division, ovum fertilization, and cell differentiation.

Not all threshold phenomena are strictly binary in nature. In many cases the magnitude of the response may be controlled by a "secondary regulator" once the primary signal pushes the response mechanism beyond threshold. Often secondary regulators such as cyclic AMP interact with responses which are mediated by Ca^{2+} acting as a primary regulator. In this case, calcium initiates the response, after which cyclic AMP modulates the strength and/or duration of the response. A different type of interaction involves secondary regulators which alter the value of calcium concentration corresponding to the threshold. (6) In this way secondary regulators may function to either enhance or inhibit cell responses to primary stimuli.

The Interaction Between Cyclotron Resonance and Biological Systems

A number of experiments have been conducted in which weak ($B_o \approx 400$mGauss), low frequency ($f < 1$kHz) magnetic fields were found to alter various biological functions. (5) Proposed explanations of these phenomena center around ions

which regulate cell functions and the effect of cyclotron resonance upon those ions. Chiabrera et al. (8) have proposed a model in which cyclotron resonance perturbs cell surface receptors to which ligands bind. McLeod and Smith (3) have proposed a model which predicts helically-shaped ion transport channels in the cell membrane. According to the latter model, ions enter and exit the cell through the channels in a fashion similar to that of a bead sliding down a helically-shaped string, as depicted in Figure 6a. The exact mechanism by which the cell controls the flow of ions is open to conjecture. One possible explanation involves the alternate compression and elongation by the cell of a helical channel along which the ion is constrained to travel. (3) Let us assume a force due to the concentration gradient and represent it as $\mathbf{F}_{ax}$ in Figure 6a. Compression of the helical channel (as shown in Figure 6b) would result in a relatively small component of $\mathbf{F}_{ax}$ acting in the direction of motion. This component is represented by F_m. The flow of ions through the channel would be relatively slow. Conversely, if the helix were elongated by the cell (see Figure 6c), F_m would be relatively large and ions would flow through the channel more rapidly. That cyclotron resonance involves circular motion and that helically-constrained motion is quasi-circular lends some credibility to the McLeod-Liboff model.



a) ion channel b) helix compressed c) helix elongated

Figure 6. The proposed ion channel model.

If some membrane channels indeed fit the McLeod-Liboff model, their helical shape, coupled with the fact that cyclotron resonance deals exclusively with circular motion, suggests that the proper cyclotron resonance condition should affect the rate of ion flow into or out of the cell and should thus provide at least some control of cell functions which depend upon intracellular ion concentration. (3) Consider, for example, a single cell in a medium containing a physiologically normal concentration of Ca^{2+}. Suppose that a single-axis, DC B-field is applied such that a Ca^{2+} ion will tend to move in a circular orbit having a radius less than or equal to the radius of the helical channel. If an AC E-field having a frequency of w_p corresponding to resonance for Ca^{2+} is applied perpendicular to B_o, the motion

of calcium ions in the channel should be affected by the applied fields. This model may be tested quantitatively.

If an organism which exhibits an ion concentration-dependent process were exposed to cyclotron resonant fields tuned to that ion, a change in the intracellular concentration of that ion should produce a corresponding change in that process. Measuring changes in that process would then provide an effective method of testing the model. McLeod et al. (5) have performed several such tests.

Experiments were performed upon the marine diatom Amphora coffeaeformis, the motility of which shows strong dependence upon the extracellular concentration of Ca^{2+}. Cultures of diatoms were placed on agar plates and exposed to cyclotron resonant magnetic fields tuned to Ca^{2+}. The motility of the exposed diatoms was compared to that of identical cultures which were not exposed. The differences in motility were found to be quite significant. (5) The exposure to resonance was found to increase the motility of the diatoms by as much as a factor of five. This experiment seems to indicate that Ca^{2+} cyclotron resonant fields can indeed interact with calcium ions to affect biological function.

EXPERIMENTAL PROCEDURES

This chapter describes the procedure of the two experiments performed for this project. The aim of the first experiment was to test the response of the proliferation of Adult T-cell Leukemia (ATL) cells to varying extracellular calcium concentration independent of the resonant fields. This is termed the calcium dose response relationship. In the second experiment, groups of the cells were exposed to calcium ion cyclotron resonant fields and their growth was compared to control groups which were grown under identical conditions but were not exposed to the resonant fields.

The cells were grown in α Minimum Essential Medium (α MEM) with 10% bovine fetal calf serum. In addition, a low-calcium version of α MEM was prepared for the experiments. When this medium was prepared, the addition of $CaCl_2$ was omitted. Prior to its addition to the low -calcium medium, calf serum was dialyzed against 12 L of 0.15N saline to remove any free calcium ions. Enough serum was then added to the medium to comprise 1% of the total volume. This medium was used to make the experimental growth medium and was also used to wash any free calcium from the cells at the beginning of experiments. To make the growth medium for experiments, calcium concentration was precisely adjusted by

adding varying amounts of supplemental $CaCl_2$ solution to the low-calcium medium. The details of the preparation of the experimental medium are discussed below.

The 6T-CEM ATL cells were received from American Type Tissue, Inc. (Bethesda, MD), and thawed at 25 degrees Celsius, then placed into a tissue culture flask with 20ml of α-MEM and incubated for 7 days at 37 degrees Celsius. By that time the medium in the flask was saturated with cells, so they were passaged, wherein the cell suspension is diluted and placed into a new flask with fresh medium to grow again to the point of saturation. The cells were then passaged every 7 days. Experiments were started at the time of passaging.

Experiment 1

After passaging, the excess cells were centrifuged and washed in low-calcium medium and centrifuged again. While the cells were spinning, the concentration series for the experiment were prepared in culture dishes. The series consisted of two 6-well culture dishes (12 wells total), with two wells for each level of calcium added to the medium. The calcium levels tested were 0, 1, 2, 3, 4, and 5 moles/liter (mmol). Each well received 3ml of low-calcium α MEM to which an amount of calcium corresponding to the position of that well in the series had been added. For example, the first two wells in a series contained low-calcium α MEM with 0 mmol

Ca^{2+} added while the second pair contained medium with 2 mmol Ca^{2+} added, etc. Recall from above that the calcium concentration was adjusted by adding precise amounts of $CaCl_2$ solution to low-calcium α MEM.

When the cells finished spinning they were counted and aliquots containing $2.5x10^5$ cells were placed into each well of the culture dishes. The dishes were then placed in the incubator at 37 degrees Celsius. After 72 hours of incubation the cells in each well were centrifuged and resuspended in 0.5ml of medium. The 0.5ml suspension from each well was then combined with 0.5ml of trypan blue stain and the live (unstained) cells were counted in a hemocytometer. The average number of viable cells in the two wells for each level of added calcium was calculated. The data obtained from Experiment 1 is presented later in the "Results" section of this paper.

Experiment 2

The aim of Experiment 2 was to test the effect of cyclotron resonant fields on ATL cell line 6T-CEM. The cells were tested for response to magnetic fields precisely adjusted to the cyclotron resonance for calcium ions. The preparation of the culture dishes for Experiment 2 was identical to that for Experiment 1 except that two identical concentration series were prepared, one experimental series and one control series. Each series consisted of two, 6-well

culture dishes (12 wells total), with two wells for each level of added calcium. Again the levels tested were 0, 1, 2, 3, 4, and 5 moles/liter (mmol).

After passaging, the excess cells were centrifuged, washed in calcium-free medium, and then centrifuged again. After the second spinning, the cells were counted and, using micro pipets, aliquots containing $2.5x10^5$ cells were placed into each well of the culture dishes. The control and experimental dishes were all placed in the same incubator. In addition, the experimental dishes were placed at the center of the coils which generated the cyclotron resonant fields. The control and exposed cells were physically separated by 12 inches, which insured that the AC magnetic field near the controls was less than 0.1 mGauss peak. After 72 hours of incubation, the cells in each well were centrifuged and resuspended in 0.5ml of medium. The 0.5ml suspension from each well was then combined with 0.5ml of trypan blue stain and the live (unstained) cells were counted in a hemocytometer. The average number of live cells in the two control wells was compared to the average in the two experimental wells for each concentration in the series. The data obtained in Experiment 2 is presented in the "Results" section of this paper.

RESULTS

This section presents the results of the project. The first section describes the developmental steps which were required to achieve viable experimental procedures. The data obtained through those procedures then follows.

The Development of the Experiment

Throughout the course of this project a number of problems were encountered which inevitably caused experiments to produce erratic data. Many changes were made to attempt to correct the problems. Artifacts were discovered and eliminated. Procedures were changed to minimize errors and simplify the interpretation of the results. The final experimental procedure as it appears above is the result of all these modifications. This section documents some of the more significant changes and the reasons for their implementation. The initial set of experiments was conducted in Cooley Laboratory (the Microbiology laboratory on the Montana State University campus). Two types of cells, Baby Hamster Kidney (BHK) cells and Human Rectal Tumor (HRT) cells were tested. After the experiments with the BHK's and HRT's failed to produce conclusive data, the project was temporarily halted to analyze and eliminate the problems in

the experiment. One major problem was the fact that the cell lines which were being used had never been tested for a response to calcium. A database search was conducted for a cell line with a documented calcium dependence. Although several suitable lines were discovered, ATL (Adult T-cell Leukemia) cells were chosen because Shirakawa et al. (9) have demonstrated a direct dependence of these cells upon calcium for growth. Another problem encountered in the Cooley Lab experiments was that a heavily-used ultracentrifuge in the laboratory sat within two feet of the cyclotron resonance coils. It was discovered that any time the centrifuge was operated during a resonant field experiment, the fields produced by the ultracentrifuge significantly altered the resonant fields from their originally calibrated values. Ultimately, that problem was solved by setting up a new laboratory in Cobleigh hall (the Electrical Engineering building) where it was possible to control the fields at all times. All subsequent experiments were then performed there.

Once the project was moved to Cobleigh Hall, an attempt was made to measure the calcium dose response relationship of the new ATL cells. A concentration series was prepared in two 6-well dishes (12 wells total). The series consisted of duplicate wells with low-calcium medium to which six different levels of calcium were added as described previously. The levels originally tested were 0, 5, 10, 20, 30, and 100% of normal for the medium. Cells were grown in

the dishes and counted. Those counts were compared in order to verify the calcium dependence of the cell line as reported by Shirakawa et al. (9) Variations in cell growth rate versus added calcium were minor and inconsistent. It was discovered that the original experimental medium was actually quite high in calcium concentration. Although dialysis effectively removed most of the free Ca^{2+} from the calf serum, it apparently contained significant amounts of protein-bound calcium. Some of this stored calcium was probably released after the serum was added to the medium, resulting in significantly high levels of Ca^{2+} in the presumably low-calcium experimental medium. Thus, the experimental medium, even with no added $CaCl_2$, was not low enough in Ca^{2+} to affect the growth rate of the cells. This problem was remedied by reducing the amount of added calf serum to 1%. The calcium dose response relationship was also improved by changing the range of $CaCl_2$ levels in the concentration series. Originally the levels of added calcium were 0, 5, 10, 20, 30, and 100% of normal for α MEM, where 100% of normal corresponds to 1.8 mmol. It was decided to expand the range of calcium concentration, changing the levels of addition to 0, 1, 2, 3, 4, and 5 mmol. This resulted in satisfactory repetition of the Shirakawa study. (8) That data is presented below in the "Experimental Results" section.

Once a calcium response was established, a problem with repeatability was discovered. Counts performed upon theoretically identical populations of cells showed significant variation. This indicated a problem with either the inoculation procedure or the counting procedure. First the inoculation procedure was changed. Populations of $2.5x10^5$ cells were added to each well in both series. Originally the wells were inoculated by micro pipets. Obtaining aliquots with $2.5x10^5$ cells required volumes of cell suspension ranging from 10 to 60 μL. It was thought that errors were probably introduced by such factors as variable amounts of suspension remaining on the pipet tip and errors inherent to the micro pipets. These possible artifacts were eliminated by preparing each experimental well with 2ml of low-calcium α MEM to which a 1ml suspension of cells plus low-calcium α MEM was to be added at a later step. To obtain an end volume of 3ml with the proper level of calcium, each of the 2ml volumes received the amount of $CaCl_2$ needed to make 3ml of medium at the desired level. Thus, when 1ml of calcium-free medium containing $2.5x10^5$ cells was added, the result was 3ml of medium with the proper level of calcium. (NOTE: The volume occupied by $2.5x10^5$ cells in 1ml of fluid was assumed to be negligible.) Thus, the cells were diluted with low-calcium medium so that 1ml of suspension contained $2.5x10^5$ cells. Each well was then inoculated with 1ml of that suspension using disposable 1ml pipets. This

change did little to improve repeatability between theoretically identical aliquots. Thus, the counting procedure was changed.

Originally, after the 72 hour incubation, the cells from each well were transferred into a 15ml centrifuge tube and spun for 10 minutes, forcing the cells into a pellet at the bottom of the tube and leaving the medium in a layer above the pellet. That layer of fluid is called the supernatant. The supernatant was then removed from the tube, leaving only the cell pellet. A 1ml aliquot of α MEM was added to the tube and the tube was vortexed to resuspend the cells. A 0.5 ml volume of the suspension was removed and combined with 0.5ml of trypan blue dye. A hemocytometer was then filled with a minute amount of this new suspension and placed under a microscope where the cells were counted.

The irregularity in the counts was probably introduced during the step in which 0.5ml of the 1ml cell suspension is removed and combined with the trypan blue dye. The 1ml suspension was probably not being made sufficiently homogeneous just prior to the removal of the 0.5ml. Thus, 0.5ml volumes taken from two different but identical suspensions may contain significantly different numbers of cells. For example, a 0.5ml volume removed from one suspension may contain 1.0×10^6 cells while an equal volume removed from an identical suspension may contain 1.8×10^6 cells. That possible artifact was eliminated by changing

that step of the counting procedure. The cell pellet was resuspended in 0.5 ml of medium instead of 1ml. That 0.5 ml of suspension was then combined directly with the 0.5ml of trypan blue dye. This eliminated any variability in that step resulting from a non-homogeneous suspension. The change also produced the secondary beneficial effect of doubling each cell count by counting all of the cells from each well rather than half of them. This produced a more reliable sample size for the counts.

The above changes have greatly improved the experimental protocol. However, at the time of the writing of this paper, experiments are providing only preliminary indications of an effect (as discussed in the sections below). Further improvements must occur before the data will be unquestionably reproducible and therefore conclusive.

Experimental Results

Calcium Dose Response of ATL Cells

First, the effect of the concentration of calcium in the culture medium on the in vitro proliferation of ATL cells was studied. Figure 7 shows the mean values of the cell counts from the four repetitions of the dose response test plotted against amount of calcium added to the low-calcium medium in millimoles per liter (millimolarity). The cell number values have been normalized to the value of the control at zero calcium concentration. The cells grow poorly in medium with

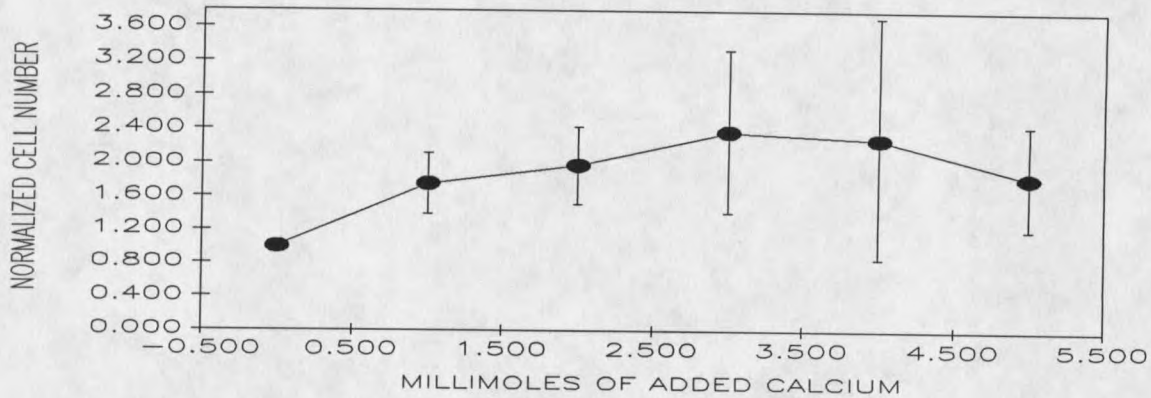
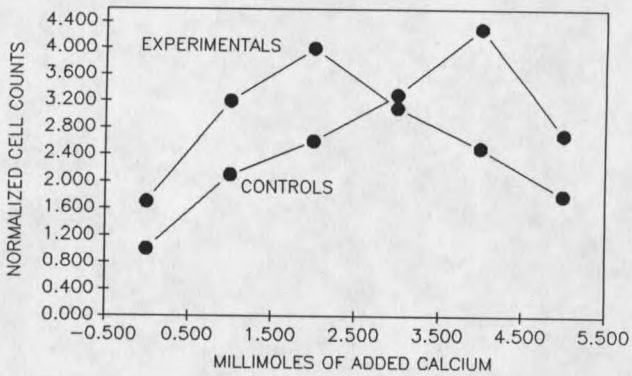


Figure 7. Averages from the four dose response experiments. Cell numbers are normalized to the value at 0mmol and bars represent σ.

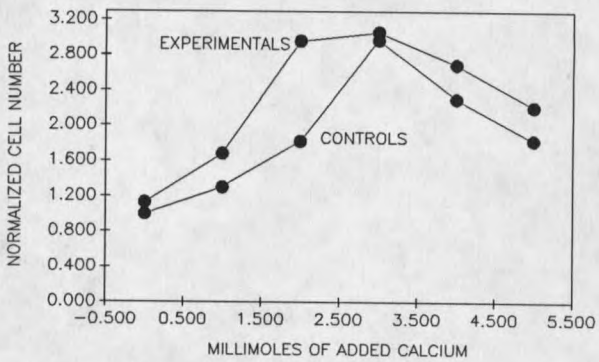
zero added calcium. Proliferation increases steadily up to a peak at a level of added calcium between two and four millimolar. As the level of calcium is increased beyond the peak value, cell proliferation drops. This agrees well with the data from the Shirakawa data [9], which indicated a peak at four millimolar.

Effect of Cyclotron Resonance upon ATL Cells

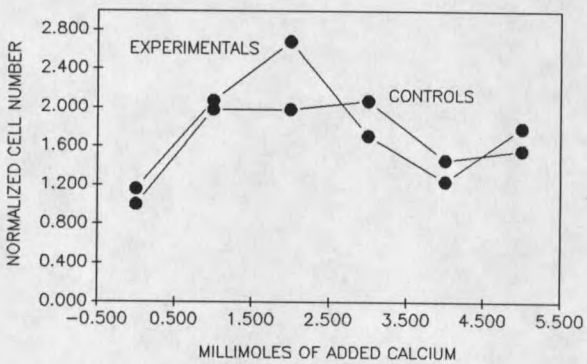
Next, the fields were tuned to calcium ion cyclotron resonance (with B_o = 209 mGauss, B_{ac} = 200 mGauss, and f_c = 16 Hz). The experimental cultures were then grown in the cyclotron resonance (CR) space, counted, and compared with counts of control cultures. Results of triplicate runs appear in Figures 8a-c.



Plot a. Plot of data from repetition 1 of the CR experiment.



Plot b. Plot of data from repetition 2 of the CR experiment.



Plot c. Plot of data from repetition 3 of the CR experiment.

Figure 8. Data plots from repetitions 1-3 of the CR experiment with cell counts in each plot normalized to the value at 0 mmol.

The curve for the experimental cultures is superimposed over the curve for the controls in each of the three plots. Again all cell counts are normalized to the value of the control at zero added calcium and plotted against added calcium. All three plots show the experimental cells proliferating faster than the control cells. At calcium levels above 4mmol, the proliferation rate of both control cells and experimental cells drops. Figure 9 shows the mean values of the counts from the triplicate repetitions plotted against amount of calcium added.

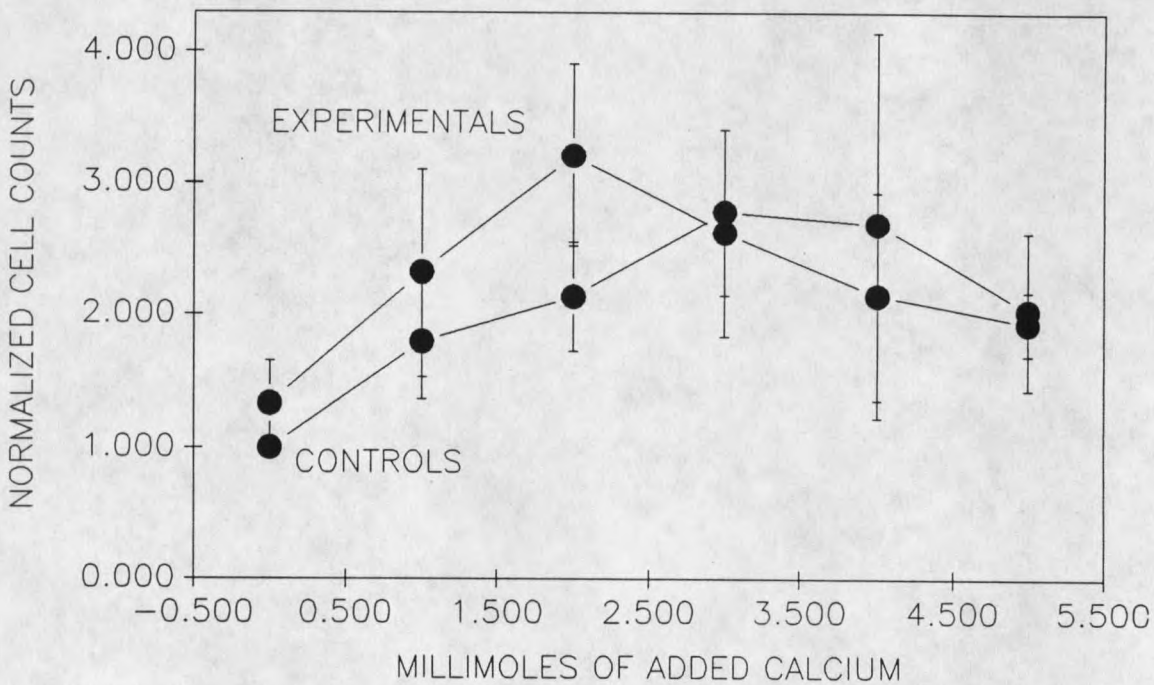


Figure 9. Plot of the averages from repetitions 1-3 of the CR experiment. Cell counts are normalized as before.

As before the cells exposed to resonance outperform the controls at the lower concentrations. Exposed cells growing

in medium with 1mmol of added calcium grow at the same rate as unexposed cells growing in medium with approximately 2.3mmol of added calcium. At higher levels of added calcium, the growth rate of both experimentals and controls drops. However, the experimentals peak near a level of two millimolar, while the controls peak near the three millimolar level. The difference in growth rate between the experimentals and the controls at lower concentrations of calcium, coupled with the difference in the position of the two peaks, suggests that the effect of cyclotron resonance is to lower the concentrations at which the normal effects of calcium upon the growth rate of ATL cells (increased growth at lower concentrations and diminished growth at higher levels) occur.

CONCLUSIONS

A preliminary experimental protocol aimed at testing the effects of cyclotron resonance upon the growth rate of Adult T-cell Leukemia cells was developed. The project produced some well-defined data on the response of the cells to extracellular calcium concentration, as well as a nearly complete framework for testing the cyclotron resonance effect and some preliminary data indicating its probable existence.

The dose response studies established that the ATL cells require extracellular calcium for growth, but that excess calcium causes a decrease in cell proliferation. The cyclotron resonance experiments indicate that the proliferation rate of the cells is boosted at lower concentrations by exposure to the calcium ion resonant fields. In addition, the peak of the experimental curve occurs at a lower concentration than that of the control curve. It seems that the fields shift the proliferation curve of the experimental cells to the left relative to the control curve. These observations indicate that the fields act to increase the calcium concentration apparent to the cells. The implication is that calcium-tuned cyclotron resonance either increases the intracellular calcium concentration as predicted by the theory presented in this

paper, increases the activity of the calcium ions inside or outside the cell, or causes some combination of those effects. Further improvements in the experimental procedure are expected to produce more consistent results. If produced, conclusive data would add to the already large body of evidence that cyclotron resonance, tuned to biologically important ions, is one mechanism of interaction between low energy electric and magnetic fields and biological systems. New test procedures will be developed to help determine whether the fields are increasing intracellular concentration or increasing the activity of ions inside or outside the cell.

This project served to develop a framework for further testing of the hypothesis through the continuation of the overall project. Most of the preliminary problems associated with the test environment and the collection of data have been solved. This study strongly suggests that the exposure of 6T-CEM ATL cells to cyclotron resonant magnetic fields tuned to the calcium ion will, either by increasing the concentration of calcium ion apparent to the cell, increasing the activity of Ca^{2+}, or a combination of both effects, cause the corresponding effects upon proliferation.

Further study is indicated to establish a frequency response curve, proliferative response to other ionic species, and the response of the ATL cells to the resonant fields when the media also contains a chemotherapeutic agent

such as cytosine arabinoside (ara-C). By injecting ara-C into the vicinity of a tumor cell and immediately exposing the tumor to calcium cyclotron resonance, the toxic effects of ara-C upon the tumor could be increased. The tumor cells, proliferating even faster than normal, would then absorb even more of the chemotherapeutic agent than normal.

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