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Synthetic Liposome and Natural Membrane  
Bilayer Systems with Direct Application to  
the Controlled Delivery of Chemical Agents**

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The Influence of Oscillating Electromagnetic Fields on Membrane Structure and Function: Synthetic Liposome and Natural Membrane Bilayer Systems with Direct Application to the Controlled Delivery of Chemical Agents.

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ABSTRACT

Investigations have been conducted to determine if an imposed electromagnetic field can influence membrane transport, and ion and drug permeability in both synthetic and natural cell membrane systems. Microwave fields enhance accumulation of sodium in the lymphocyte and induce protein shedding at  $T_c$ . Microwaves also trigger membrane permeability of liposome systems under specific field exposure conditions. Sensitivity varies in a defined way in bilayers displaying a membrane structural phase transition temperature,  $T_c$ ; maximal release was observed at or near  $T_c$ . Significantly, liposome systems without a membrane phase transition were also found to experience permeability increases but, in contrast, this response was temperature independent. The above results indicate that field-enhanced drug release occurs in liposome vesicles that possess a  $T_c$  as well as non- $T_c$  liposomes. Additional studies extend non- $T_c$  liposome responses to the in vivo case in which microwaves trigger Gentamicin release from a liposome "depot" placed subcutaneously in the rat hind leg. In addition, evidence is provided that cell surface sequestered liposomes can be triggered by microwave fields to release drugs directly into target cells. This "microinjection" technique as well as the general release of drugs from an in vivo liposome "depot" may form the basis of an effective technique for controlled delivery of drugs.

INTRODUCTION

Cells interact with their environment first through the cell membrane. For example, the translocation of charged species across both natural and synthetic membrane systems and the binding of extracellular agents leads to transduction of signals to internal sites within the cell. Such events involve structural changes in the organization of the bilayer. Involvement of specific constituents such as receptor sites and ion channels are crucial in this process. Given the major role that the cell membrane plays in signal transduction it is important to understand how the membrane bilayer responds to an imposed electric or magnetic field.

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The underlying hypothesis for studies in our laboratory is that membrane structure and conformation is important for coupling to external fields (1-3). External fields employed in the studies discussed here are time-varying electromagnetic fields in the microwave frequency range. Such fields interact with biological systems according to how well energy is transferred to components of the target membrane system. For microwave fields, membrane structures that are dipolar, such as polar amino acid side chains and cell-surface associated bound water, will undergo field orientation at microwave frequencies.

In previous studies employing the erythrocyte we have shown that sodium cation permeability is enhanced during exposure to 2450 MHz fields in a striking, temperature-dependent manner (1,4). Microwave-enhanced sodium transport was observed at 17 - 19.5C which is linked to the presence of a membrane structural phase transition known to exist in the erythrocyte bilayer at 17 - 19.5C. Addition of cholesterol to the membrane, which eliminates Tc, rendered the bilayers unresponsive to microwaves. The increase in permeability at Tc was linearly dependent on the electric field strength, reversible, enhanced by oxygen, and reduced by the presence of antioxidants. Importantly, the later findings raise the possibility that free radical species may be operative in this interaction. Three other laboratories have independently confirmed a permeability increase at Tc in the erythrocyte during microwave exposures (6 - 8).

Further studies in our laboratory employing polyacrylamide gel electrophoresis (PAGE) have identified structural changes associated with the permeability increase at Tc (2,4). At least eleven protein species ( $< 31,000$  Da) are shed or released from the erythrocyte in response to microwave treatment at Tc. Interestingly, microwave-induced protein shedding was enhanced by oxygen and most prominent for 28,000 - 31,000 and  $< 14,000$  Da species. Significantly, these protein species are also released from the erythrocyte membrane by chelating calcium and this strongly suggests that cationic salt bridge formation may be destabilized by microwaves.

Protein shedding has been recently confirmed by us using quantitative high performance liquid chromatography (HPLC)(5). Protein shedding involved the release of approximately 450 fgm of protein per erythrocyte; this corresponds to 1% of the total protein mass of the cell, but to greater than 50% of the protein mass of the cell membrane or stroma. By employing different interactive HPLC columns for protein separation, protein species released were found to be relatively nonpolar, hydrophobic, and to carry a net positive electrostatic charge. The class of proteins shed are most likely loosely bound peripheral or extrinsic proteins associated with the exterior of the cell surface. Such proteins play a major role in the transduction of signals to integral proteins which span the bilayer.

These studies raised the important question of whether proteins, phospholipids, or both are participating in this membrane response. To determine if phospholipids in membranes are influenced by fields a series of liposome experiments were conducted in our laboratories (9 - 12). Employing either microwave or static magnetic fields exposure of liposome vesicles was found to increase significantly as a function of temperature, with maximal release occurring at or near  $T_c$  for phase transition vesicles. In the presence of microwave fields this effect was linearly dependent on the electric field strength, enhanced by oxygen, and reduced by the presence of antioxidant agents. This response is similar to that displayed by the erythrocyte. In the presence of static magnetic fields a permeability increase was observed at or near  $T_c$  but the effect was nonlinearly dependent on magnetic field strength; a threshold for response was observed with an  $ED_{50}$  of 15 mTesla and a 95% confidence limit of 6.5 - 34.9 mTesla. These studies indicate that membrane bilayers constructed purely of phospholipid components are responsive to microwave and static magnetic fields. We have developed a model for this interaction in which membrane deformation plays a major role in destabilizing the bilayer and leads to membrane permeability changes (12).

In this report we present new data extending previous microwave studies in two important ways. First, the lymphocyte has been examined for microwave sensitivity by testing for alterations in sodium permeability and transport and for alterations in protein shedding. This eukaryotic cell type is significantly different from the erythrocyte since it is nucleated and its cell membrane is responsible for a wide variety of transport and receptor binding functions. Secondly, liposome studies have been extended to include a characterization of the microwave sensitivity of non-phase transition liposomes; both in vitro and in vivo studies are presented. In addition, we present data characterizing the transfer of chemical agents from liposomes bound at the surface a target cell to the interior of the target cell. The latter protocol constitutes the basis for an effective "microinjection" technique.

## EXPERIMENTAL METHODS

Source and Preparation of Lymphocytes and Liposome Vesicles. Spleen lymphocytes from young adult male Sprague-Dawley rats (250 gm) were harvested as described (19). Mixed suspensions of lymphocytes and erythrocytes were fractionated by centrifugation through Ficoll-Hypaque density gradients. Viability of cell preparations before and after microwave or sham treatments were comparable and exceeded 95 - 97% viability, as judged by nigrosin dye exclusion. Cells were maintained in phosphate buffered Ringer's solution, pH 7.4, containing 11 mM glucose throughout.

Large unilamellar liposomes (LUVs) were used throughout and

prepared as described (9). All phospholipids were of ultrahigh purity and acquired from Avanti Polar Lipids, Inc., Pelham, AL, USA: DPPC, dipalmitylphosphatidylcholine; DPPG, dipalmitylphosphatidylglycerol; PC, egg lecithin; PE, lysinylphosphatidylethanolamine; aT, alpha-Tocopherol. DPPC:DPPG vesicles were formed at a 1:4 mole ratio; DPPC:PE, PC:PE vesicles were at a 95:5 mole ratio; PC:DPPG:aT vesicles were at a 90:9.9:0.1 mole ratio. All liposome systems had a final phospholipid concentration of 33 mg/ml. Vesicles were loaded with 6-carboxyfluorescein (6-CF) at self-quenching concentrations of >100mM, or with Gentamicin, USP grade, at 25mg/ml. Triton X-100 was used at 1% (v/v) to lyse liposomes and establish 100% maximal release. Release of 6-CF was monitored by an increase in fluorescein fluorescence of the sample (492nm EX, 518nm EM), and release of Gentamicin into rat blood was monitored by performing plasma determinations using a RIA kit available from NEN, Billerica, MA, USA, Gentamicin[I-125] RIANEN Cat. # NEA-075.

Lymphocyte Sodium Transport Assays and PAGE Analysis. Sodium transport assays were as described (1), with cells at  $5 \times 10^6$ /ml. Ouabain treatment was at 0.1 mM and carried out at 25C for 60 minutes. Polyacrylamide gel electrophoresis was performed as described previously (2). Dilution series for the sham-treated samples was at >50, 20, 15, 10 ug/well; microwave treated samples were loaded at 10 ug/ml. Sensitive silver staining was used to visualize epsilon-amino and SH-groups of proteins. Gel standards were phosphorylase B (91 kDa), BSA (68 kDa), OVA (45 kDa), carbonic anhydrase (31 kDa), and lysozyme (14 kDa).

In Vitro Target Cell "Microinjection" Experiments and In Vivo Liposome "Depot" Studies. For target cell studies lymphocyte-liposome complexes were formed by incubating cells at  $5 \times 10^7$  in phosphate buffered Ringer's solution, pH 7.4, containing 11 mM glucose with liposomes at 1.5 mg phospholipid/ml at 25C for 30 minutes with gentle shaking. Formed complexes were rapidly washed 5X (12,000 rpm, 5sec in an Eppendorf microfuge) with buffer at 4C to remove any unbound 6-CF. The supernatant was monitored for 6-CF and none was detected in the fifth wash. Lymphocytes after liposome binding were at greater than 95% viability. Following either sham or microwave treatment complexes were rapidly washed 3X and the complexes were resuspended and assayed for the presence of 6-CF spectrofluorometrically.

In vivo studies involved placing a subcutaneous liposome "depot" in the hind quarter of a Sprague-Dawley rat 24 hours prior to use. Liposomal Gentamicin loading was approximately 14 mg/kg; liposomal injections remain localized and formed a well-defined circular depot. The 24 hour pre-treatment period enabled any free, extraliposomal Gentamicin to clear the blood stream prior to the experiment. On the day of the experiment animals were anesthetized, and, employing atropine sulfate to reduce nasal secretions, the carotid artery was cannulated. At times before,

during, and after microwave or sham treatment 300 ul of blood was withdrawn and replaced with an equal volume of isotonic saline. Animal temperature was maintained by using a heating pad set at 38C. Core body temperature was monitored throughout using a Yellowsprings thermistor probe inserted into the rectum. At 20 minutes following anesthesia the animals were stabilized with respect to core body temperature (37C) and respiratory rate (60 - 80 bpm).

In Vitro and In Vivo Microwave Exposures. In vitro exposures of lymphocytes, liposomes, and lymphocyte-liposome complexes were performed at 2450 MHz employing a device described in detail (1,2, 9). This system is capable of controlling and monitoring sample temperature, input and output power, sample mixing, and, if desired, sample pO<sub>2</sub>, pN<sub>2</sub>, pCO<sub>2</sub>. In vivo microwave exposures were localized at the site of the liposome "depot." Microwave treatments (2450 MHz) were performed using a 0.75 inch contact applicator available from ELMED Corp., Addison, IL, USA. SAR, specific absorption dose rate in mw/gm, was computed as described using a value of 1.2 kcal/°C for the specific heat of lymphocyte suspensions, and 0.83 kcal/°C for the specific heat of rat tissue (1). SAR values for in vitro exposures were 6 - 8 mw/gm throughout. SAR values for in vivo exposures were 6 mw/gm.

## RESULTS

Arrhenius Analysis of Active and Passive Transport of the Sodium Cation in Rat Spleen Lymphocytes. Previous studies conducted in our laboratory indicate that the erythrocyte displays a structural transition at temperatures in the range of 17.0 - 19.5C (1,4); importantly this is where microwave sensitivity has been observed by us (1,2,4) and others (6 - 8). To determine if rat spleen lymphocytes display a membrane structural phase transition an Arrhenius plot was constructed for both passive and active sodium transport. Identification of T<sub>c</sub> is associated with a break in the slope of the Arrhenius plot and this temperature would be a candidate for microwave sensitivity experiments. Figure 1 displays these data and we observe that a population of mixed spleen lymphocytes exhibit a maximum value for passive accumulation of sodium (Ouabain-treatment) in the lymphocyte at a temperature of 37C. Total sodium accumulated (untreated cells) reaches a minimum at 37C. Thus, the difference, which corresponds to accumulation of sodium due to active transport, achieves a maximum at 37C, as shown in Figure 1. Since the pump extrudes sodium this means pump activity is minimized at 37C. This may be an adaptive advantage associated with the physiological temperature of 37C. Taken together the data indicate that these spleen lymphocytes exhibit a break at 37C in Arrhenius plots for both passive and active sodium transport. We are not aware of any previous studies characterizing the temperature dependence of sodium transport in the lymphocyte.

Microwave Exposures Facilitate the Accumulation of Sodium in the Lymphocyte During Passive and Active Transport at Tc. The Arrhenius analysis presented in Figure 1 indicates that spleen lymphocytes exhibit a Tc at 37C. To determine if microwave fields influence active and passive transport of sodium in the lymphocyte at Tc microwave exposures were performed at 37C and at 40C. Figure 2 presents these data and no significant differences were observed between lymphocytes treated with microwaves at 40C and control samples maintained at 40C. When exposures were conducted at 37C, however, an approximately two-fold increase in sodium accumulation during passive transport was observed in response to microwave treatment. Sodium accumulation in the lymphocyte during active sodium transport was also increased approximately 1.6 fold. Passive transport data indicates that sodium entered the cell in greater quantities in the presence of the microwave field; this is interpreted as a permeability effect. Active transport data indicates that sodium accumulated in the cell and this is an indication of pump inhibition. This suggests that significantly longer, chronic exposures might lead to an irreversible accumulation of sodium in the lymphocyte.

Protein Shedding From the Lymphocyte Occurs at Tc. Previous studies from our laboratory employing the erythrocyte indicate that the phenomenon of protein shedding occurs predominantly at Tc, and, moreover, that microwave-induced enhancement of protein shedding is most pronounced for exposures at Tc (2 - 5). It would be of interest to determine if a eukaryote cell such as the lymphocyte which displays a Tc at 37C (Figure 1) also responds to microwave fields by undergoing protein shedding. To conduct these experiments mixed spleen cell populations were incubated at 37C for 90 minutes in the presence of a microwave field as for the transport studies, or at 37C in the unenergized microwave waveguide. Cell samples were removed and rapidly spun in an Eppendorf microfuge for 5 seconds (12,000 rpm) and the cell-free supernatant was collected for analysis. Polyacrylamide gel electrophoresis (PAGE) analysis was performed and the proteins bands were silver stained for visualization.

Figure 3 depicts a typical PAGE electrophoretogram with sham-treated samples in lanes 1 - 4 (20, 15, 10, and >50 ug/ml sample dilution) and microwave-treated samples in lanes 6 - 9 (all 10 ug/ml). Both microwave and sham treatments yielded a variety of proteins spanning the molecular weight range of 91,000 to 14,000 Da that were resolved by PAGE. Two noticeable differences, however, were apparent between the microwave and sham-treated groups. First, protein species shed at molecular weights < 14,000 Da are more pronounced in the microwave-treated samples. Second, there is a unique triplet band at 44,000 Da that is apparent only in the microwave-treated samples. These results indicate that a relatively short exposure to microwave fields at Tc results in the protein shedding from the lymphocyte and that the microwave-induced pattern is different from that due to an equivalent temperature treatment.

Microwaves Fields Facilitate the Release of 6-CF From Liposome Vesicles: Tc and Non-Tc Vesicles. Our original observation that microwaves can act to release the drug cytosine arabinofuranoside from Tc liposomes was made with liposome vesicles that carried a net negative surface charge (9). These vesicles were constructed from DPPC:DPPG phospholipids in a 1:4 mole ratio. We report here that these studies have been extended in two important ways. First, Tc liposomes have been employed that carry a net positive surface charge to test whether the permeability effect is dependent on electrostatic charge. Second, non-Tc liposomes have been used to test if the permeability effect is uniquely restricted to liposome bilayers that undergo a structural phase transition. The latter test has important consequences for the generality of microwave-induced membrane permeability.

Figure 4 depicts the solute release profiles for the three model liposomes employed plotted as a function of temperature. The solute marker in each liposome system is 6-carboxyfluorescein (6-CF). Solute release was determined at each temperature after a 10 minute incubation by measuring 6-CF fluorescence and comparing it to maximal release induced by Triton-X100. DPPC:DPPG liposomes carry a net negative surface charge and have a Tc at approximately 40C. In the absence of microwaves Tc is characterized by a dramatic increase in permeability that is first detected at 40C (DPPC:DPPG, Figure 4). DPPC:lysiny-PE vesicles carry a net positive charge and also exhibit a nominal Tc at 40C (DPPC:PE, Figure 4). Notice, however, that the release of 6-CF begins at 36C since the addition of lysiny-PE broadens the phase transition region. Finally, PC:lysiny-PE (PC:PE, Figure 4) also carries a net positive charge but this liposome is in the fluid phase and does not display a phase transition over this temperature range. This is indicated by a baseline release of approximately 7 - 9% detected at all temperatures studied.

When these three liposome systems are characterized for 6-CF release in the presence of a microwave field (10 minute treatment at the temperature indicated) the permeability of each liposome was influenced. The two Tc liposomes (MW/DPPC:DPPG and MW/DPPC:PE, Figure 4) respond to the microwave field in a temperature-dependent manner with enhanced release at Tc and a broadening of the solute release profile. For example, 6-CF release was detected at 37C which is 3C below Tc where release is normally first detected. The positively charged vesicles (MW/DPPC:PE) displayed an even greater broadening with solute release detected at temperatures as low as 31C which is 9C below the nominal Tc. The non-Tc vesicles (PC:PE, Figure 4) responded in a different manner with enhanced solute release at all temperatures studied and in a temperature-independent manner. This response is characterized by a release of solute at levels of 30 - 40% of maximal release at all temperature.



Microwaves Facilitate the Transfer of Liposomal 6-CF into Lymphocyte Target Cells: Tc and Non-Tc Vesicles. The above findings indicate that microwaves can act to release an aqueous marker from both Tc and non-Tc liposome vesicles. It is of interest to determine whether liposome vesicles bound to a target cell surface can still respond to microwave fields and undergo enhanced solute release. If such an interaction does occur it could form the basis of a controlled "microinjection" technique in which liposomal solute is transferred directly into a target cell.

In these experiments purified rat spleen lymphocytes were complexed in vitro with the three liposome systems described above. Each liposome vesicle type was found to bind electrostatically to the lymphocyte surface in significant quantities so that fluorescent measurements of 6-CF release was possible. Lymphocyte-liposome complexes thus formed were thoroughly washed and subsequently treated with a microwave field or sham exposed at either 24C for 15 minutes or at 34C for 30 minutes. These temperatures were chosen since they were well below the nominal Tc of 40C for the Tc liposomes (Figure 4). The complexes were then extensively washed and the amount of 6-CF transferred into the lymphocyte target cell was assayed spectrofluorometrically. In Figure 5 is depicted the results of these experiments. Each of the vesicle systems, both Tc and non-Tc, were influenced by the microwave field. DPPC:DPPG vesicles exhibited the greatest increase in 6-CF transfer to the target lymphocyte with a 8.6 fold and 8.2 fold increase of 6-CF transfer at 25C and 34C, respectively. The least responsive vesicles were the non-Tc liposomes with a 1.96 and a 1.41 fold increase in 6-CF transfer at 25C and 34C, respectively.

Microwave Triggered Release of Liposomal Gentamicin: In Vivo Test with a Non-Tc Liposome Vesicle. The finding that PC:lysiny-PE (PC:PE, Figure 4), a non-Tc liposome vesicle, exhibited enhanced release of 6-CF in response to microwave fields suggested that non-Tc vesicles may be effective in drug release experiments. To test this hypothesis the marker drug Gentamicin was loaded into PC:DPPG:aT (90:9.9:0.1) liposome vesicles. These vesicles are temperature insensitive in that they do not undergo a dramatic increase in drug release as temperature is increased; they respond to temperature as the PC:PE vesicles shown in Figure 4 with a baseline release of approximately 7 - 9% of maximal release at 37C. Prior to the in vivo experiment it was necessary to confirm microwave sensitivity of the PC:PG:aT vesicles. This characterization revealed that the vesicles respond to microwaves, under conditions as shown in Figure 4 at 37C, with enhanced drug release of approximately 45 -50% of maximal release.

In vivo experiments were performed by placing a liposome "depot" subcutaneously in the hind leg of the rat, and, 24 hours later, following blood levels of Gentamicin at times before, during, and after localized microwave treatment of the "depot."

Figure 6 depicts the typical time course of Gentamicin release from the liposome "depot" in an animal that received no microwave field exposure (sham-treated) and in an animal carrying a liposome "depot" treated with microwave fields. Sham-treated animals displayed a baseline blood concentration of Gentamicin of approximately 0.14 ug/ml. These levels correspond to times 24 hours after liposome "depot" injection. Microwave-treated animals received a localized exposure to microwaves at the site of the liposome depot at a dose rate of 6 mw/gm for 15 minutes beginning at time zero shown in Figure 6. Tissue temperature measurements using a VITEK probe, which is nonabsorbant in a microwave field (1), revealed that tissue temperature excursions in excess of 0.5C were not apparent. As shown in Figure 6 the application of the microwave field lead to a marked increase of Gentamicin in the blood compartment. At 20 minutes after the onset of microwave treatment blood concentrations of Gentamicin had risen to 0.36 ug/ml and this represents a 2.57 fold increase over baseline levels of 0.14 ug/ml. At latter times blood concentrations of Gentamicin continued to rise such that at 160 minutes blood concentrations had achieved 0.79 ug/ml; this represent a 5.64 fold increase over baseline levels.

## DISCUSSION

The results of these studies extend previous observations from our laboratory (1 - 5, 9) and, others (6 - 8), that microwave fields can act to alter membrane transport and ion permeability properties of both natural cell membranes and of synthetic liposome systems.

The research we report here adds to this previous body of literature in several important ways. First, it extends our understanding of interactions with natural cell membranes to the lymphocyte. This important cell type exhibits a Tc at 37C and this is where microwave alterations in both active and passive cation transport were observed. In addition, first steps in the characterization of microwave-induced protein shedding for the lymphocyte are presented. Secondly, studies employing liposomes have extended microwave interactions to Tc liposomes carrying a net positive charge, and, importantly, to non-Tc liposome systems. The latter is important in terms of interaction mechanisms since Tc is not involved in this response. Two other observations have been made that are of practical importance. Using both Tc and non-Tc liposomes we have demonstrated that liposomes sequestered on the surface of a target lymphocyte cell, when treated with microwave fields, can directly "microinject" a solute marker into the target cell. In addition, when a non-Tc liposome system loaded with Gentamicin is placed in a subcutaneous "depot" in vivo and treated locally with microwaves a rapid release of drug occurs and the plasma concentration of drug can rise approximately 5-fold.

The observation that microwave fields influence both passive

and active sodium transport in the lymphocyte at 37C indicates that relatively short term exposures of 90 minutes can alter lymphocyte membrane function. Alterations in sodium transport are linked to important intracellular activities (13, 20 - 23). For example, sodium/potassium transport is critically involved in cell volume homeostasis, intracellular enzyme function, membrane transport processes involving receptor binding and gate triggering, and the regulation of cellular growth and differentiation. In the latter case, an increase in sodium/potassium pump activity is one of the earliest measureable responses of B-lymphocytes to antigen stimulation, and this is essential to B-cell maturation and antibody production.

In our experiments an increase in the passive accumulation of sodium was observed during microwave treatment at Tc. This effect would lead to an increase in intracellular sodium. When active transport itself was examined during microwave treatment an elevation in sodium accumulation in the lymphocyte was detected. This means that the Na/K pump which extrudes sodium was inhibited and this would lead to elevated intracellular sodium. Interestingly, Allis (6) recently reported in a careful study that 2450 MHz microwaves significantly inhibits by 35% the Na/K pump of human erythrocytes when treated at Tc. This raises the important possibility that the sodium pump in other cell types, in addition to the erythrocyte and lymphocyte, may be inhibited by microwave fields.

We have observed protein shedding in the lymphocyte during microwave exposures at Tc. This phenomenon was first reported by us for the erythrocyte and it is an irreversible process (2 - 5). In the lymphocyte, however, the regeneration and recycling of plasma membrane proteins occurs as a natural process (14). Our studies demonstrate that both microwave and sham-treated cells display a wide range of molecular weight proteins that are shed during incubation at Tc for 90 minutes (Figure 3). Microwave treatment, however, leads to the release of a unique protein band at 44,000 Da and to the additional release of unique bands at approximately 14,000 Da. At present the significance of the loss of these protein species is not known. Little research is available on the characterization of protein shedding in the lymphocyte or any nucleated cell. Limited literature is available on blood storage alterations but such studies deal with alterations associated with erythrocytes maintained at 4C. One reference for human lymphocytes does report that overnight storage (18 hours) at 4C leads to a marked decrease in the percentage of cells staining positive with anti-Leu2a (suppressor cells), which resulted in a significant depression of the measured ratio of helper to suppressor T lymphocytes (15). This effect was not observed if storage was at 22C - storage at 37C was not reported. Recently a 44,000 Da calcium-binding protein was isolated from the lymphocyte cytoskeleton by Owens and Crumpton (16, 17). The 44,000 Da protein that is released from the lymphocyte by microwaves at Tc might be related to this protein. At present an immunospecific antibody for its identification, however, is not yet available.

The notion that phospholipids bilayers can interact with microwave fields in the absence of intergral proteins was tested in these studies with several different liposome systems. Phase transition liposomes carrying either a net negative or a net positive charge underwent microwave-induced permeability increases at pre-Tc temperatures, where they are not normally leaky (Figure 4). It is not known why positively charged liposomes responded to the microwave fields with greater release at temperatures further removed from Tc.

Perhaps the most significant liposome findings are that non-Tc vesicles are responsive to microwaves. Here we report that two non-Tc systems respond to microwaves by experiencing increased membrane permeability (PC:PE of Figure 4 and PC:DPPG:aT of Figure 6). The bilayers of both of these liposomes are fluid at temperatures employed in these experiments. Our interpretation of Tc liposome interactions with microwave fields depends on the presence of Tc (9). We hypothesized that microwave fields induced rotational motion in the phospholipid acyl chains, or the polar head groups, similar to that experienced at Tc, to trigger pore formation and permeability increases. Since the addition of 30 mole % cholesterol to the bilayer, which abolishes Tc, has been shown by us to eliminate microwave sensitivity we reasoned that the ability to undergo a phase transition at Tc was critical. This interpretation is appropriate for Tc vesicles but what interaction mechanism(s) explains our non-Tc results? Both of the non-Tc vesicle systems we employed are fluid and it is possible that the addition of cholesterol to 30 mole % could render the bilayer unresponsive to microwaves, as it had for Tc vesicles. In both cases a solid, rigid bilayer would not be able to couple with the oscillating microwave field. This would be consistent with our original working hypothesis. Future experiments are directed at testing whether fluid bilayers that respond to microwaves are rendered microwave-insensitive by the addition of cholesterol.

Two important applications of microwave-triggered liposomal drug release are presented here and they both employ non-Tc liposomes: "microinjection" of drugs into target cells, and systemic drug delivery from an in vivo liposome "depot." "Microinjection" of drugs directly into target cell populations (Figure 5) should be possible in vivo by targeting liposomes using antibodies directed to specific cell surface markers (18). The second application of controlled, systemic delivery of drugs into the blood stream is possible through microwave-triggered release of drugs from a liposome "depot" (Figure 6). Both of these approaches complement existing methodologies for drug delivery. At present a key problem to liposomal drug delivery is the step of actually triggering the transfer of drug to a target cell, or, alternatively, triggering the release of the drug from the liposome for systemic distribution. A particularly attractive aspect of using microwaves to actively control drug release from liposome systems is that this modality is noninvasive.

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## FIGURE LEGENDS

Figure 1: Temperature Dependence of Sodium Transport in the Rat Spleen Lymphocyte. An apparent structural transition is present at 37C. Na-22 Flux represents accumulation of sodium in the cell after 90 minutes. Mean +/- S.D. (n = 9).

Figure 2: Effect of Microwave Fields on Sodium Transport in the Lymphocyte: Influence of Exposures at  $T_c$ . Na-22 Flux represents accumulation of sodium in the cell after 90 minutes. Passive and active transport as in Figure 1. Microwaves exposures at 2450 MHz, SAR = 7 mw/gm, 90 minutes. Sham exposures in unenergized waveguide, 90 minutes. Mean +/- S.D. (n = 9).

Figure 3: Protein Shedding in the Lymphocyte at 37C ( $T_c$ ). Microwave (M) and Sham (S) treatments were as in Figure 2, and cells were rapidly centrifuged and the cell-free supernatant was analyzed by PAGE. Lanes 1 - 4: Sham at 20, 15, 10, > 50 ug/well. Lanes 6 - 9: 10 ug/well. Standards referred to in text.

Figure 4: Effect of Microwave Fields on the Release of Carboxyfluorescein From Liposome Vesicles: Influence of  $T_c$  and non- $T_c$  Liposome Systems. Microwave (MW) treatment was at an SAR of 6 mw/gm for 10 minutes at the temperatures indicated. Unexposed samples were temperature treated for 10 minutes in the unenergized waveguide. Dye release was measured immediately thereafter.  $T_c$  Liposomes: DPPC:DPPG, DPPC:PE. Non- $T_c$  Liposome: PC:PE. Maximal release of dye was achieved with 1% Triton X-100.

Figure 5: Microwave-Triggered "Microinjection" of Liposomal Carboxyfluorescein into Lymphocyte Target Cells: Influence of  $T_c$  and Non- $T_c$  Liposome Systems. Microwave (EM) exposures of liposome-lymphocyte complexes were at an SAR of 7mw/gm for the times and temperatures indicated. Sham exposures were at identical temperatures in an unenergized waveguide.  $T_c$  Liposomes: DPPC:DPPG, DPPC:PE. Non- $T_c$  Liposome: PC:PE.

Figure 6: Microwave-Triggered Release of Gentamicin From an In Vivo Liposome "Depot" into the Blood Circulation. Microwave treatment was at an SAR of 6 mw/gm for 15 minutes; increments in tissue and core body temperature over 0.5C were not detected. Blood was obtained via a catheterized carotid artery and plasma levels of Gentamicin were determined using an RIA assay.

Figure 1

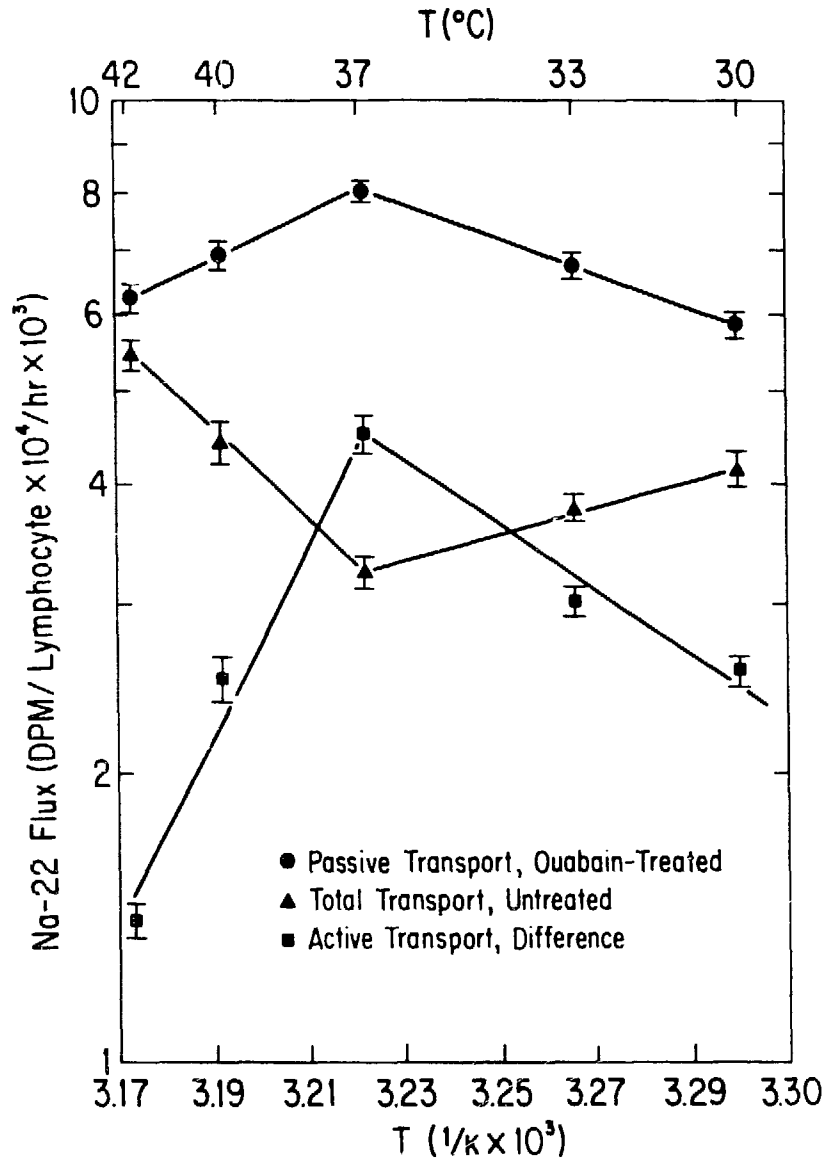




Figure 2

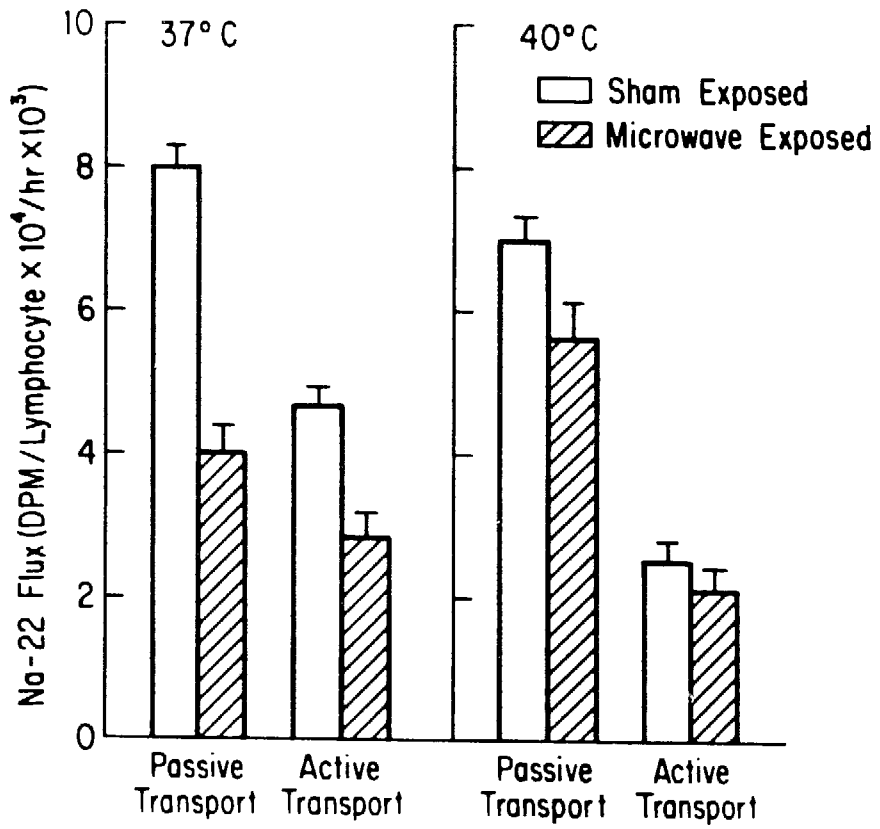
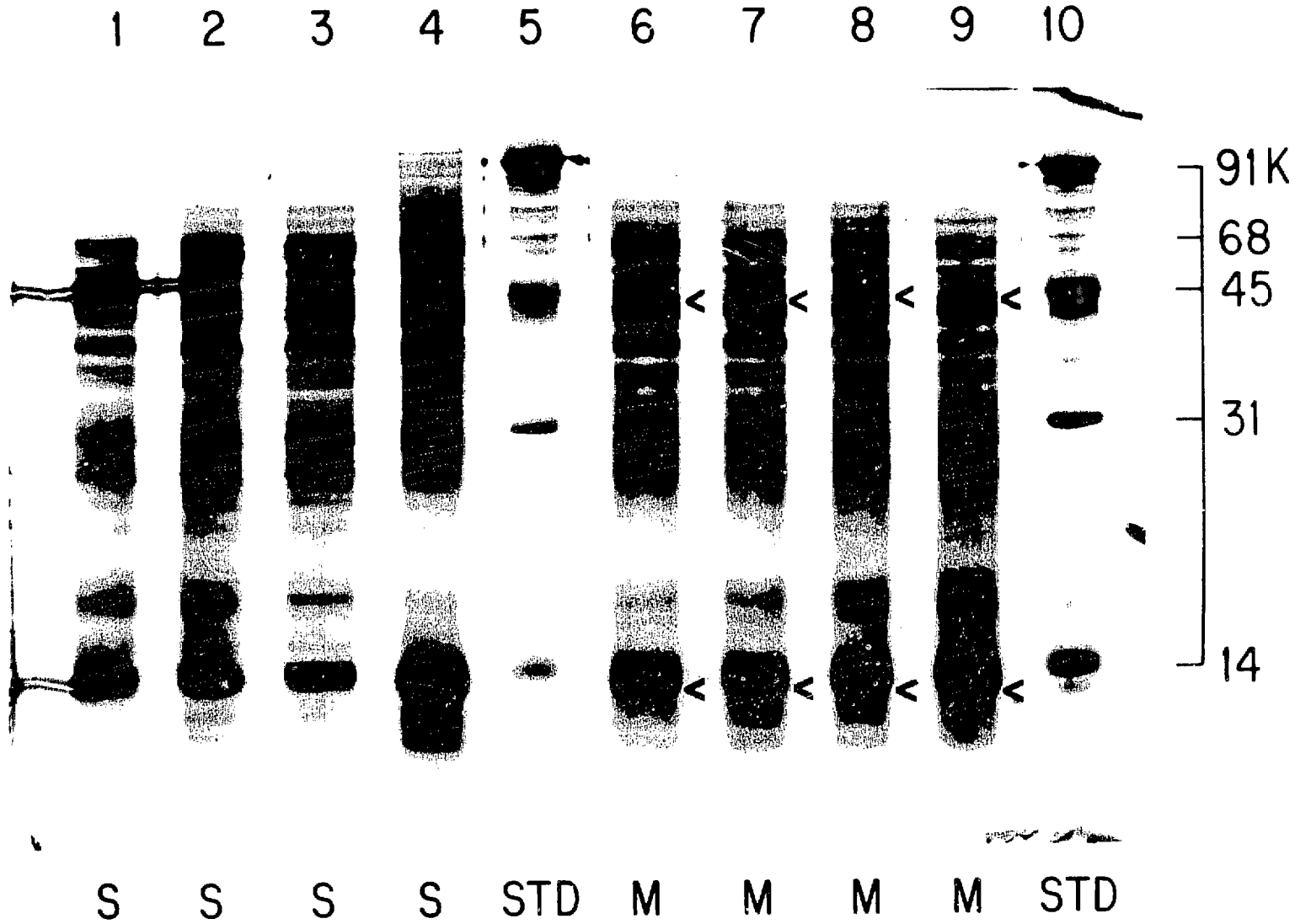


Figure 3



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Figure 4

# FIELD-TRIGGERED DYE TRANSFER IN LIPOSOME:LYMPHOCYTE COMPLEXES

## Spectrofluorometry

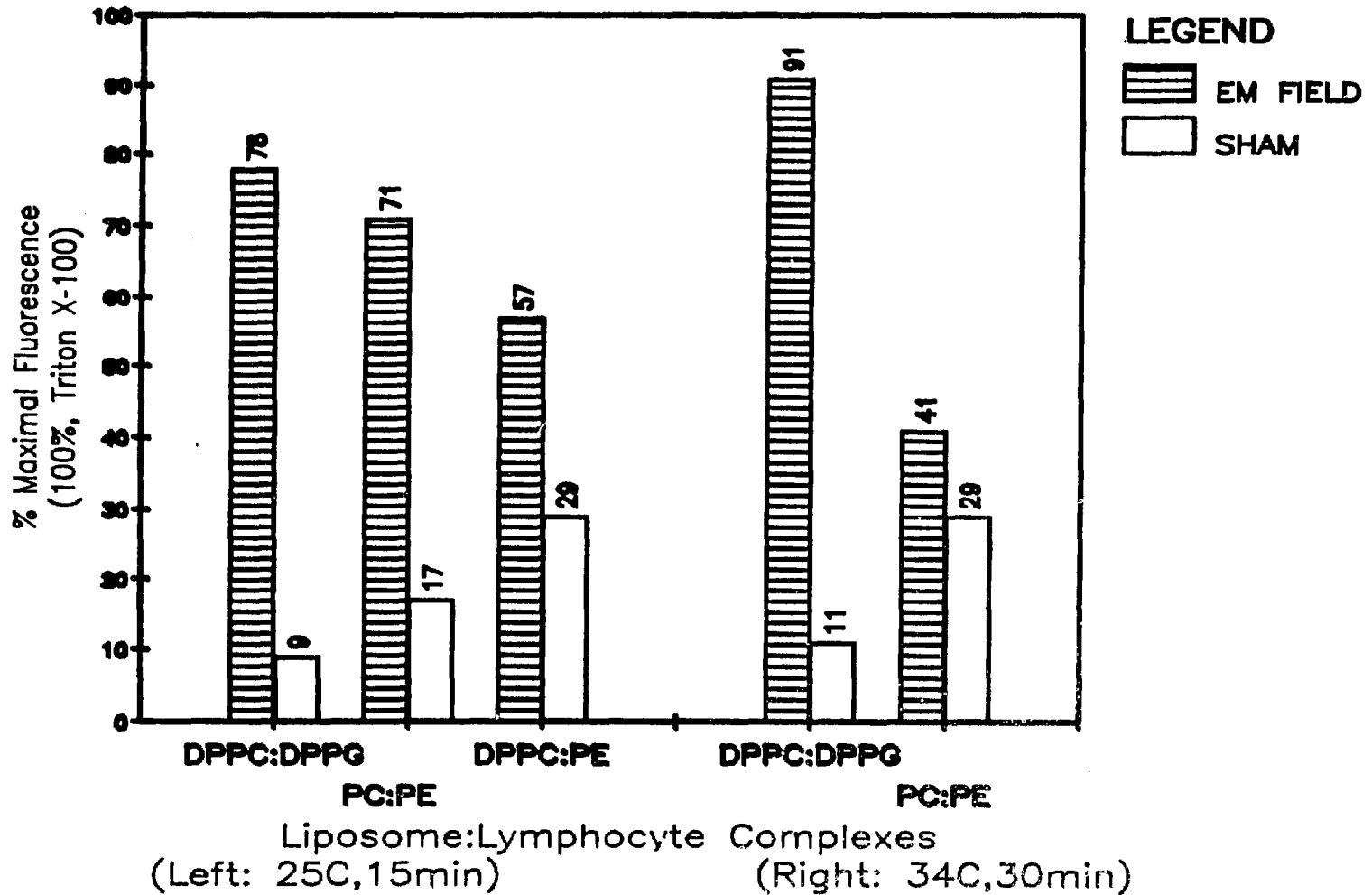


Figure 5

# LIPOSOME DYE RELEASE: Microwave vs. Sham Heating

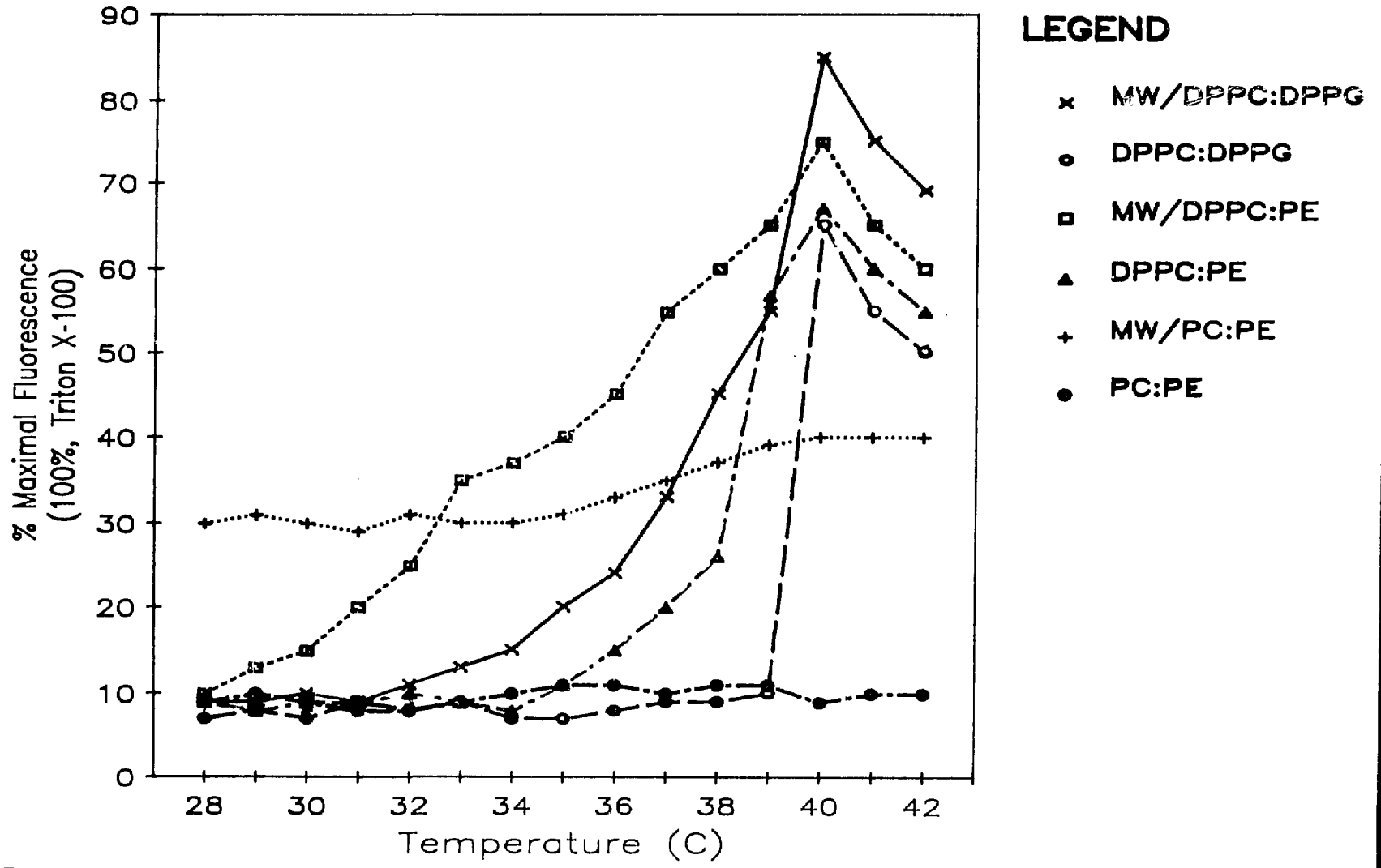


Figure 6

### MICROWAVE TRIGGERED GENTAMICIN RELEASE:

Single Microwave Treatment

