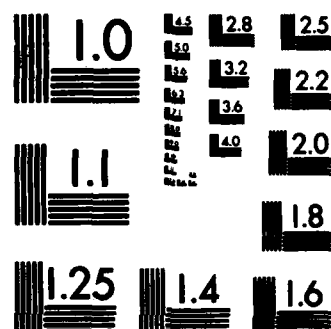AD-A142 979   EFFECTS OF MICROWAVES ON CELL MEMBRANE PERMEABILITY(U)   1/1
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Microwave Radiation	Protein Shedding	Peripheral Proteins	Nonionizing Electromagnetic Radiation
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Membrane Permeability	Free Radicals	Erythrocyte	Transition
Cation Transport	Antioxidants	Oxygen	Membrane Phospholipids
Arrhenius Analysis	Liposomes	Plasma Factors	Poly Acrylamide Gel Electrophoresis

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

The objective of this research project was to identify and characterize cell membrane responses to microwave radiation and, importantly, to determine specific conditions or modulators required for these responses. This study has revealed that membrane permeability changes in the erythrocyte and in liposome vesicles, as well as protein shedding in the erythrocyte, are induced by microwaves at the membrane phase transition, and that these responses are strongly

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dependent on plasma, oxygen tension, and antioxidant free radical scavengers. These findings provide new insight into both the physical and chemical nature of microwave radiation interaction with the cell membrane.

Erythrocyte Studies - Microwaves (2,450 MHz, CW) have been shown to increase sodium permeability in rabbit erythrocytes for exposures only within a narrow temperature range of 17-19°C (T_c) which coincides with the membrane phase transition. Sodium permeability is not increased when the cell membrane is cholesterol-loaded. Since cholesterol eliminates the phase transition at T_c, this result directly links the microwave effect to the membrane phase transition. The microwave affect at T_c is a nonlinear function of absorbed power (SAR, mW/g) but a linear function of the associated internal electric field, E_{IN} (V/m). At 400 mW/g and 23 V/m the microwave effect saturates. The permeability increase was found to be reversible and transient in that immediately following termination of exposure, sodium permeability was decreased but returned to normal within 60 minutes.

Plasma, oxygen, and antioxidants are extracellular factors that were found to exert a significant influence on the microwave effect. The presence of plasma profoundly potentiates the increase in sodium permeability at T_c. Oxygen also modulates the microwave effect with relative hypoxia (5 mm Hg), characteristic of intracellular pO_2, and hyperoxia (170 mm Hg) enhancing the permeability increase. Significantly, the presence of either ascorbic acid or mercaptoethanol, which are both antioxidants and effective free radical scavengers, completely inhibit the microwave effect.

Structural changes involving protein shedding from the erythrocyte membrane have also been identified in response to microwave exposure. Examination of cell-free supernatants from microwave-treated erythrocytes by polyacrylamide gel electrophoresis and sensitive silver staining has revealed at least nine proteins (<28,000 D) being shed or released in response to microwaves. Protein shedding occurs only at T_c and results in release of 24,000 and 26,000 D proteins only visible in microwave-exposed erythrocytes. In contrast, microwave treatment resulted in a pronounced shedding of 28,000, 15,000, 14,400, 13,000, 11,000, 10,000, and 8,000 D proteins also seen in sham-treated cells. Furthermore, protein shedding was observed to be markedly sensitive to pO_2 with relative hypoxia and hyperoxia enhancing the effect. This correlates with the pO_2 sensitivity observed for microwave increased cation permeability. Additional studies have demonstrated that protein shedding is not a result of activated autoproteolytic activity and does not involve membrane disruption and the release of integral proteins. Studies have established that calcium-chelators simulate microwave-induced protein shedding; this strongly suggests that protein shedding involves the release of peripheral or extrinsic proteins which are loosely bound to the cell surface via cationic bridges.

Liposome Studies - Microwaves (2,450 MHz, CW) have been shown to stimulate the rapid release of a chemotherapeutic drug from liposome vesicles. In this work large unilamellar liposomes were prepared from two completely saturated and highly purified phospholipids by the reverse evaporation process with cytosine arabinofuranoside (ARA-C, 243 MW) encapsulated in the interior aqueous phase. These liposomes are not permeable until heated to ~40°C (T_c) when a phase transition results in near complete drug release without liposome membrane disruption.

(CONTINUATION PAGE - Robert P. Liburdy, N00014-81K-0669)

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That microwaves stimulate drug release in liposome membranes devoid of proteins demonstrates that phospholipids per se are important targets for microwave radiation. The strong pO_2 dependence, potentiation by plasma, and inhibition by free radical scavengers observed suggest a chemical basis of interaction. Based on these findings liposomes should provide a useful model for studying interaction mechanisms between electromagnetic radiation and biological membranes.

FINAL REPORT

Effects of Microwave on Cell
Membrane Permeability

ONR N00014-81-K-0669

1 July 1981 - 30 June 1984

Principal Investigator: Robert P. Liburdy, Ph.D.

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I.   SUMMARY OF WORK ACCOMPLISHED

Introduction

This final report summarizes the important findings of work accomplished over years 01, 02, and 03.   Detailed research findings for year 03 are provided in the attached three manuscripts.   Annual reports 01 and 02 have presented detailed descriptions of earlier completed work.

ATCH 1:   "Microwave bioeffects in the erythrocyte are temperature and pO_2 dependent: Cation permeability and protein shedding occur at the membrane phase transition".   R.P. Liburdy and A. Penn, Bioelectromagnetics 5, 283-291 (1984).

ATCH 2:   "Microwaves and the cell membrane.   II. Temperature, plasma, and oxygen mediate microwave-induced membrane permeability in the erythrocyte".   R.P. Liburdy, P.F. Vanek, Jr.   Submitted to Radiation Research.

ATCH 3:   "Microwave stimulated drug release from liposomes".   R.P. Liburdy, R.L. Magin, Submitted to Science.

Summary

The objective of this research project was to identify and characterize cell membrane responses to microwave radiation and, importantly, to determine specific conditions or modulators required for these responses. This study has revealed that membrane permeability changes in the erythrocyte and in liposome vesicles, as well as protein shedding in the erythrocyte, are induced by microwaves at the membrane phase transition, and that these responses are strongly dependent on plasma, oxygen tension, and antioxidant free radical scavengers.   These findings provide new insight into both the physical and chemical nature of microwave radiation interaction with the cell membrane.

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That microwaves stimulate drug release in liposome membranes devoid of proteins demonstrates that phospholipids per se are important targets for microwave radiation. The strong pO_2 dependence, potentiation by

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II. INDEX OF PUBLICATIONS AND PRESENTATIONS

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Liburdy, R.P. and Vanek, P.F., Jr. Microwaves and the cell membrane. II. Temperature, plasma, and oxygen mediate microwave-induced membrane permeability in the erythrocyte. Radiation Research, Submitted for publication.

Microwave Bioeffects in the Erythrocyte Are Temperature and pO₂ Dependent:
Cation Permeability and Protein Shedding Occur at the Membrane Phase Transition

R.P. Liburdy and A. Penn

Institute of Environmental Medicine, New York University Medical Center, New York

Microwave exposure (2450 MHz, 60 mW/g, CW) of rabbit erythrocytes increases Na passive transport only at membrane phase transition temperatures (T_c) of 17-19 °C. This permeability effect is enhanced for relative hypoxia which is characteristic of intracellular oxygen tension ($pO_2 \leqslant 5$ mm Hg). Neither the permeability nor the pO_2 effects are observed in temperature-matched ($\pm$ 0.05 °C), sham-exposed controls. In addition, at T_c, microwave exposure is observed to induce the shedding or release of two erythrocyte proteins not seen in sham-exposed controls. Moreover, the enhanced shedding of at least seven other proteins all of molecular weight $\leqslant$ 28,000 D was detected in the microwave-treated samples. Using sensitive silver staining we estimate that approximately 450 fg of protein were shed per erythrocyte. These results demonstrate that temperature and pO_2 are important influences on both functional and structural responses of cell membranes to microwave radiation.

Key words: microwaves, Na transport, oxygen tension, erythrocytes, membrane phase transitions, protein shedding

INTRODUCTION

The cell membrane is widely regarded as a biological structure likely to be responsive to microwaves [Illinger, 1981]. For erythrocytes, the most consistently reported effect is an increase in cation permeability over values for sham-exposed cells during irradiation at room temperatures [Baranski et al, 1974; Olcerst et al, 1980; Fisher et al, 1982; Cleary et al, 1982]. Increases in cation permeability have not been observed for microwave exposures performed at or near 37 °C [Hamrick and Zinkl, 1975; Liu et al, 1979; Peterson et al, 1979]. A membrane phase transition was recently suggested as a possible basis for this permeability increase [Cleary et al, 1982; Fisher et al, 1982; Liburdy, 1983], but has not yet been demonstrated. Here we report that microwave-induced increases in Na-22 permeability in the rabbit erythrocyte are directly linked to a membrane phase transition, T_c, that occurs at 17.7-19.5 °C.

In previous experimentation on erythrocyte permeability, and on cellular responses in general, investigators have not controlled pO_2. Since pO_2 varies consider-

ably in vivo, we have designed experiments to simulate in vivo values of oxygen tension during microwave exposures. We report that at T_c, microwave-induced Na-22 permeability is further enhanced when oxygen tension is reduced from 150 mm Hg (atmospheric pO_2) to 0–5 mm Hg (intracellular pO_2).

In an effort to identify changes in erythrocyte protein composition occurring during microwave-induced changes in cation permeability, we also examined cell-free supernatants from exposed cells for evidence of protein shedding. We have employed a relatively new, ultrasensitive, silver-staining procedure to visualize protein resolved by SDS-electrophoresis on polyacrylamide gels. We report that microwaves induce the release or shedding of at least nine proteins ($\leq$ 28,000 D) at T_c, and that this effect is strongly dependent on oxygen tension.

EXPERIMENTAL PROCEDURES

Preparation of Erythrocytes and the Na-22 Uptake Assay

Erythrocytes from four adult male New Zealand White rabbits were used in these studies. Erythrocytes were obtained from one animal per experiment and used for both microwave and sham-exposure treatments; data shown represent the mean $\pm$ SD for individual animals obtained from N experiments. Erythrocytes were used at 1×10^{10} cells/ml in Ringer's solution (pH 7.40) with 11 mM glucose and 0.01% bovine serum albumin (BSA) present. Rabbits were bled from the marginal ear vein at staggered intervals of 30 days, and less than 10% of total blood volume was removed during each bleed. Erythrocytes were washed three times with buffered Ringer's at 4 °C. Care was taken each time to remove and discard the top layer of cells to eliminate any leukocyte contamination. For the Na-22 uptake assay, 1.4 ml of leukocyte-free, 3× washed cells were pretreated with 0.1 mM ouabain for 90 min at 37 °C and then placed in a teflon exposure tube that was thermojacketed with a dielectric coolant. Teflon as well as dodecane or DOW 200, used as coolants, possess low static dielectric constants with a relatively flat frequency response and are essentially transparent to microwaves [Weast, 1973]. Immediately prior to sham or microwave exposures, 4 μCi of Na-22 per ml cells were added and after a 10-, 20-, or 30-min exposure, cells were rapidly washed three times (7,000 $\times$ g, 10 s) at 4 °C. Na-22 uptake was linear for both microwave and sham-exposed cells during this period. To quantitate Na-22 uptake, 100 λ of washed, packed cells were counted in a gamma-well scintillation system. In all experiments, a stream of premixed O_2/N_2 was gently bubbled (0.09 LPM) into the sample tube to maintain erythrocytes in suspension and to control oxygen tension. This bubbling also aided in establishing a uniform temperature throughout the sample. Direct pO_2 measurements were made before and after microwave exposures using a thermostated Clark oxygen electrode. Intracellular (0–5 mm Hg), venous (35 mm Hg), arterial (90 mm Hg), atmospheric (150 mm Hg), and hyperoxic (760 mm Hg) conditions were simulated. Erythrocytes were equilibrated at pO_2 values for 20 min prior to onset of Na-22 influx measurements. In no case were differences in hemolysis observed between temperature-matched microwave and sham-exposed samples. Hemolysis was assessed by assaying for hemoglobin present in the cell-free supernatant using the cyanmethemoglobin method (Sigma Technical Bulletin No. 525, Sigma Chemical Co, St. Louis). Hemolysis increased slightly with temperature; a maximum of 2% of available intracellular hemoglobin

was released at 25 °C. Variations in pH of greater than $\pm$ 0.01 units were not detected.

Microwave Exposures

Microwave exposures were performed at 2,450 MHz (CW) (0–60 mW/g) in a tunable, standard waveguide section designed to permit continuous measurement of forward and reflected power [Rabinowitz et al, 1977]. In addition, simultaneous monitoring of sample temperature was performed during exposures using a nonperturbing VITEK thermistor probe ($\pm$ 0.01 °C) [Bowman, 1976]. A Teflon exposure tube that accepted approximately 1 ml of erythrocyte suspension (6 mm ID, 30 mm long) was placed in the center of the waveguide parallel to the E-field vector during microwave exposures. The specific absorbed dose rate (SAR, mW/g) was determined from the rate of temperature rise measured during exposure of a nonthermostatted suspension of erythrocytes [Peterson et al, 1979]. For 60 mW/g the rate of temperature increase of the cell suspension was 0.86 °C/min. Different steady-state exposure temperatures were achieved by adjusting the temperature of the dielectric coolant circulating around the Teflon exposure tube. Sham exposures were conducted with the microwave waveguide unenergized and with the sample temperature in the Teflon exposure tube maintained by the circulating dielectric coolant. We employed premixed O_2/N_2 to maintain cells in suspension, as well as to control pO_2. This feature eliminated thermal gradients greater than $\pm$ 0.05 °C in the exposure volume as detected by the Vitek probe; thermal hot spots can lead to erroneous microwave effects owing to spurious thermal gradients [Galvin et al, 1981]. As an additional precaution, microwave exposures were conducted at atmospheric pO_2 using a Teflon stirring rod to determine if the gentle bubbling of the O_2/N_2 gas mixture influenced microwave effects on permeability and protein shedding. No difference was noticed between stirring and bubbling at atmospheric pO_2. In addition, it was determined by microscopic examination that erythrocytes do not adhere to the surface of bubbles, thus eliminating possible confounding effects due to an erythrocyte-air interface.

Sodium Dodecyl Sulfate (SDS) Polyacrylamide Gel Electrophoresis and Silver Staining

Immediately after microwave or sham exposures, samples were centrifuged at 7,000 × g for 10 s (4 °C). Protein content of the cell-free supernatants was determined using bovine serum albumin (BSA) as a standard [Lowry et al, 1951]. Electrophoresis on 12.5% acrylamide slabs in the presence of SDS [Laemmli, 1970] was followed by silver staining [Wray et al, 1981]. Each well was loaded with 20 μg of protein which included 1.0 μg of BSA present in the supernatant to serve as an internal standard. Slabs were silver-stained for 10 min and developed for 15 min. The molecular weight markers used were: lysozyme (14,400 D), carbonic anhydrase (31,000 D), ovalbumin (45,000 D), bovine serum albumin (68,000 D), and phosphorylase B (92,000 D).

RESULTS

Figure 1 shows the temperature dependence of Na-22 influx for microwave and sham-exposed cells. The Arrhenius plot for sham treatment reveals two straight lines with a nonlinear region between 17.7 and 19.5 °C, T_c. Erythrocytes from a variety of sources are reported to exhibit such a region at temperatures within the range of

T (°C)

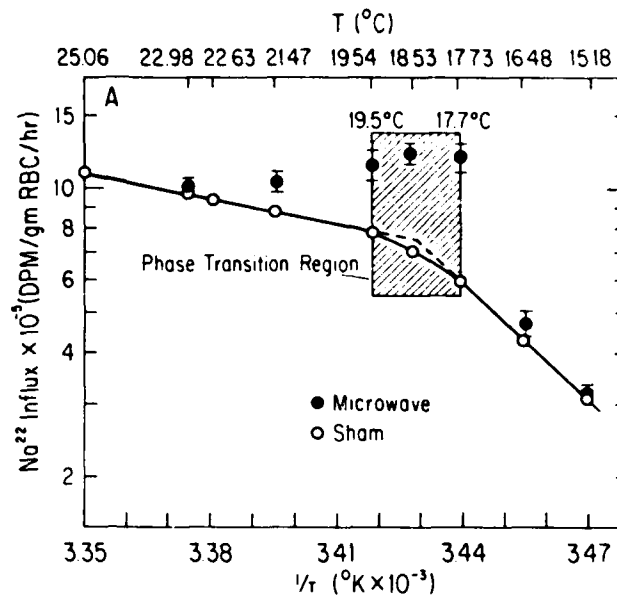25.06 22.98 22.63 21.47 19.54 18.53 17.73 16.48 15.18

Fig. 1. Arrhenius plot of Na-22 influx in the erythrocyte. Microwave exposures were conducted at 60 mW/g (2,450 MHz, CW), atmospheric pO₂, for 30 min. Temperature was monitored during exposures and cells were maintained in suspension by gently bubbling air at 0.09 LPM. Data points represent the mean ± SD. N = 6.

18–20 °C [Morris and Clarke, 1981]. The difference in slope of the two lines corresponds to an approximate doubling of activation energy as temperature is decreased (6.5 kcal/mole vs 14 kcal/mole). This change in activation energy is indicative of a protein-lipid or lipid-lipid phase transition [Elford, 1975; Silvius and McElhaney, 1982]. The broad phase transition we observe (~ 1.5–2.0 °C) is characteristic of natural membranes and is unlike the sharp transitions of 0.1–0.2 °C observed for two-component phospholipid bilayers [Moore et al, 1981].

For microwave exposures, the Arrhenius plot in Figure 1 reveals that an increase in Na-22 influx of 75–100% occurred only at T_c. The small temperature fluctuations measured in the bulk solvent during microwave exposures (≤ 0.05 °C) do not account for this large increase in influx over that in sham-exposed cells at T_c. An examination of the Arrhenius plot indicates that at 17.7 °C a temperature increase of approximately 10 °C is required for a 100% increase in Na-22 influx for sham-exposed cells. The Arrhenius plot constructed here for rabbit erythrocytes identifies only one phase transition region between 15 and 25 °C. That microwave-induced increases in permeability occur only at 17.7–19.5 °C indicates that this effect is linked to T_c.

One previous study has used an Arrhenius analysis to examine Na-22 permeability in the rabbit erythrocyte during microwave exposure [Olcerst et al, 1980]. Microwave absorption (> 60 mW/g; 2,450 MHz, CW) was associated with an increase in Na-22 permeability at 22.5 °C where the Arrhenius plot showed an unusual vertical "zigzag"; however, the activation energies above and below 22.5 °C were unchanged. The latter indicates that a phase transition was not operative; such a zigzag feature has not been previously reported in the literature and is not readily

interpretable. We have been unable to reproduce these findings using the same procedures and apparatus employed by Olcerst. In contrast, in the experiments reported here, cells were continually maintained in suspension to avoid settling and the formation of thermal hot spots. Moreover, we have measured sample temperature directly during microwave exposures; in the previous study, estimates of sample temperature were calculated on the basis of circulating coolant temperature. Factors relating to erythrocyte settling, which are significant at 30 min, thermal hot spots, and the lack of direct real-time temperature measurements may account for the results of this earlier study.

In the past, microwave exposures of erythrocytes have been conducted at atmospheric pO_2 (150 mm Hg) which is far from in vivo intracellular values in tissue (<30 mm Hg) [Johnson, 1963]. Therefore, the role of pO_2 in mediating the microwave effect on permeability was studied. A typical dose-response curve for exposures at 17.7 °C is shown in Figure 2. Here, pO_2 was varied to simulate intracellular, venous, and arterial oxygen tension. In addition, pO_2 levels corresponding to atmospheric and hyperoxic conditions were tested. At oxygen tensions corresponding to intracellular pO_2 (0–5 mm Hg) and to relative hyperoxia (760 mm Hg), the microwave-induced increase in Na-22 influx was further amplified. This effect was least pronounced at arterial and atmospheric pO_2. Significantly, sham-exposed cells were not affected by changes in pO_2. In addition, oxygen tension did not influence the Na-22 influx of cells exposed to microwaves at non-T_c temperatures (not shown). These
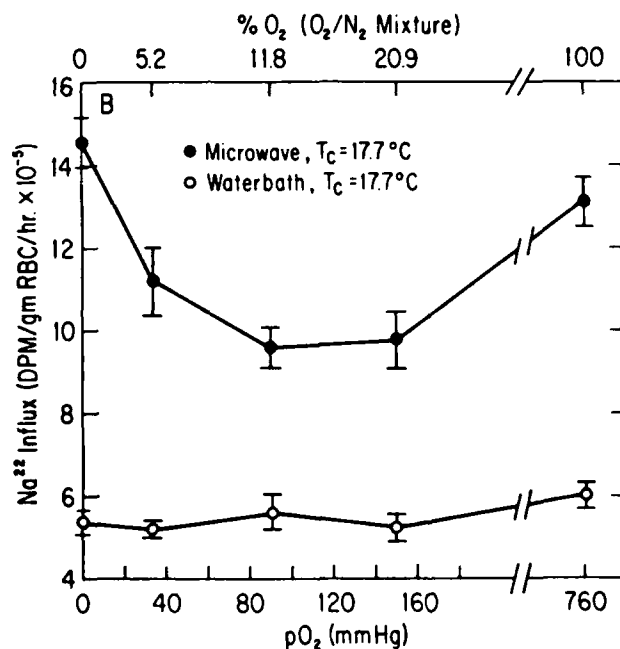

Fig. 2. Effect of oxygen tension on microwave-induced cation permeability at T_c = 17.7 °C. Microwave exposures were conducted at 60 mW/g (2,450 MHz, CW) for 30 min. Oxygen tension was maintained during exposures by gently bubbling (0.09 LPM) premixed O_2/N_2 through the cell suspension. Data points represent the mean ± SD. N = 6.

results indicate that pO_2 is an important factor modulating the microwave permeability increase observed at T_c.

To identify an alteration in protein composition in response to microwaves at T_c, the release or shedding of proteins from erythrocytes was also investigated. Figure 3 demonstrates that prominent bands (MW $\leqslant$ 28,000 D) appear in the cell-free supernatants of microwave- and sham-exposed samples for treatments at 17.7 °C. Two bands at MW 26,000 and 24,000 D are visible only in the microwave-treated samples. In addition, microwave-treated samples display pronounced staining of proteins of MW 28,000, 15,000, 14,400, 13,000, 11,000, 10,000, and 8,000 D. In contrast, proteins of MW 20,000 and 18,000 D stain less intensely in microwave-
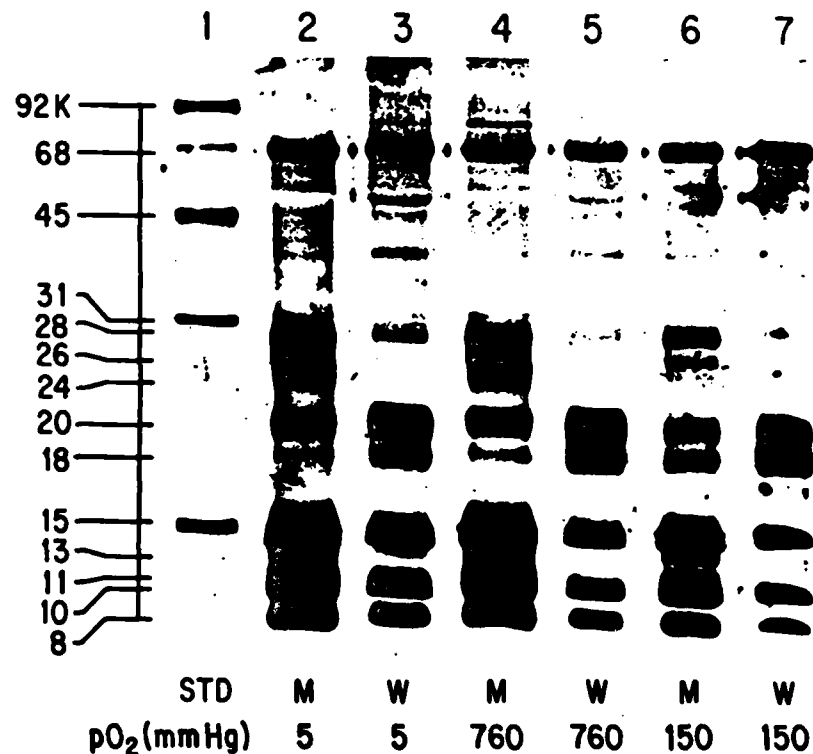

Fig. 3. Sodium dodecyl sulfate (SDS) gel electrophoretograms of proteins shed from erythrocytes. Microwave and sham exposures were as described in Figures 1 and 2. Lanes 3, 5, and 7 represent sham-exposed samples maintained at pO_2 values of 5, 760, and 150 mm Hg, respectively. Lanes 2, 4, and 6 are pO_2-matched samples treated with microwaves. Twenty micrograms of protein from cell-free supernatants was placed on each lane. Five proteins with molecular weights of ~ 18,000-28,000 D are shed by microwave-treated cells. The 26,000 D and 24,000 D proteins are visible only in the supernatant from microwave-treated cells. The 28,000 D protein appears in both samples but is more prominent in microwave-treated preparations. The other two proteins appear in both samples but are more prominent in the water-bath-treated preparations. Six lower MW proteins 15,000, 14,400, 13,000, 11,000, 10,000, and 8,000 D are shed from all samples, and all stain much more prominently in the microwave-treated samples. Hemoglobin chains correspond to MW 14,400 D. Standards used are lysozyme (14,400 D), carbonic anhydrase (31,000 D), ovalbumin (45,000 D), bovine serum albumin (68,000 D), and phosphorylase B (92,000 D). Lanes containing this solution of standards (BioRad, Richmond, CA) had a total of 5 μg of protein present.

exposed samples. Since an identical amount of protein (20 μg) was loaded onto each lane, these results suggest that a differential release occurred. This differential release involves at least 11 proteins and was not detected when microwave exposures were conducted at non-T_c temperatures in the range 15–25 °C. We estimate that we detect, as an upper limit, the release of approximately 450 fg of protein per rabbit erythrocyte; this corresponds to about 1% of the total protein mass, membrane and cytosolic, found in the human erythrocyte [Harris and Kellermeyer, 1972]. Our visualization of small amounts of protein in the cell-free supernatant was made possible by silver staining; standard Coomassie blue staining is 100-fold less sensitive.

The bands we observe are distinct from major erythrocyte membrane proteins [Todo et al, 1982], with the possible exception of the band at MW 28,000 D which may represent band 7 protein. Since a specific channel and an associated carrier protein responsible for Na transport have not been identified [Lew and Beauge, 1979], it is not possible to relate the release of proteins to the increase in Na-22 influx that we observe. It is notable, however, that protein shedding is most pronounced at pO_2 values of 5 and 760 mm Hg (lanes 2 and 4), where Na-22 influx is also most pronounced (Fig. 2).

DISCUSSION

It has been reported that in cell membranes protein-phospholipid clusters coexist with free phospholipid, and, at T_c, the clusters are thought to extrude protein toward the cell surface [Wunderlich et al, 1979]. Since microwaves are selectively absorbed by molecular dipoles such as bound water and polar amino acid side chains of proteins [Schwan, 1978], extrusion of proteins at T_c might result in a pattern of energy transfer distinct from that operating at other temperatures. That membrane proteins may be primary targets for microwave radiation is suggested by the recent demonstration that erythrocyte membrane lipids, as judged by microviscosity, are not influenced by microwave fields (1,000 MHz; 15 mW/g; 15–40 °C) [Allis and Sinha, 1981]. During our microwave exposures, care was taken to monitor temperature of the bulk solvent phase, and, owing to mixing and to thermojacketing of the Teflon exposure tube, exposure temperatures were ± 0.05 °C throughout the sample. This effectively rules out gross thermal effects as being involved in the permeability changes we observe, but leaves open the possibility of microheating of the cell membrane itself. For example, since the cell membrane contains distinct domains that are relatively hydrophilic and lipophilic, particularly in regions containing protein/lipid complexes, these areas may represent microregions with different absorbing characteristics reflecting different regional dielectric properties.

Specific mechanisms responsible for the pO_2 dependence of Na-22 influx at T_c and the shedding or release of proteins are, at present, not known. During membrane phase transitions, an abrupt change in the vertical diffusion of molecular oxygen is observed [Kusumi et al, 1982]. Also, hemoglobin is reported to associate at the erythrocyte membrane with band 3 protein [Eisinger et al, 1982]. Influx of Na-22 might be influenced either by changes in oxygen translocation or by oxygen-hemoglobin binding at the membrane. In our studies, both microwave and sham-exposed erythrocytes are intact and appear morphologically normal when examined by light microscopy. This indicates the absence of gross membrane disruption, and suggests that the proteins released are most likely the peripheral or extrinsic proteins associated

with the membrane surface. In the erythrocyte, these proteins are not intimately associated with membrane lipid, do not insert and span the bilayer, and are loosely bound to the membrane leaflet via cationic bridges [Fairbanks et al, 1971; Tanner, 1979]. Alternatively, it is possible that the proteins we detect are of intracellular origin and have leaked out from the erythrocytes during microwave and sham exposures. We do detect some hemoglobin present in the cell-free supernatant of all samples, and the band at 14,400 D corresponds to the mass of the hemoglobin subunit. Further studies will be required to determine if specific proteins are of membrane or intracellular origin. Significantly, erythrocyte proteins have not yet been studied using sensitive silver staining, and specific release or shedding of proteins from the red blood cell as a function of temperature and pO_2 has not yet been characterized to our knowledge (manuscript in preparation).

CONCLUSION

In the past, biological effects of microwaves on nonexcitable cells have been attributed in great part to *heating* regardless of the *temperature* at which exposures are conducted. The results we report demonstrate that microwave exposure at T_c is critical for inducing biological responses in the erythrocyte membrane. Specifically, the pO_2-dependent permeability change and the release or shedding of proteins we have observed indicate that both functional and structural alterations are linked to exposure at T_c. Although it is not known whether these responses can be generalized to other cells or to the case for in vivo exposures, these results provide insight into microwave interactions with the cell membrane.

ACKNOWLEDGMENTS

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ATTACHMENT 2

TITLE: Microwaves and the Cell Membrane. II. Temperature, Plasma, and Oxygen Mediate Microwave-Induced Membrane Permeability in the Erythrocyte

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PROPOSED RUNNING HEAD: Microwaves and the Cell Membrane

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LIBURDY, R.P. Microwaves and the Cell Membrane. II. Temperature, Plasma, and Oxygen Medidate Microwave-Induced Membrane Permeability in the Erythrocyte. Radiat. Res.

Microwaves (2,450 MHz) are shown to increase Na-22 permeability of rabbit erythrocytes for exposures only within the narrow temperature range of 17.7 to 19.5oC (T_c) which coincides with the membrane phase transition. Significantly, this membrane response is not observed for cholesterol-loaded erythrocytes which do not exhibit a membrane phase transition at T_c. The permeability increase at T_c is a nonlinear function of absorbed power but is a linear function of the internal electric field strength of the sample and saturates at approximately 400 mW/g and 23 V/m, respectively. The permeability increase was found to be reversible and transient in that immediately following termination of exposure sodium influx is significantly reduced but returns to normal within 60 minutes.

Extracellular factors exert a significant influence on the microwave effect. The presence of plasma markedly potentiates the increase in Na-22 permeability at T_c. Oxygen also modulates the microwave effect with relative hypoxia (5 mm Hg) and hyperoxia (760 mm Hg) enhancing the permeability increase. In contrast, the presence of two antioxidants, ascorbic acid or mercaptoethanol, inhibit the effect.

These findings raise important questions about the physical and chemical nature of microwave interactions with cell membranes and also shed light on earlier studies reporting either positive or negative effects on membrane permeability.

INTRODUCTION

The cell membrane is considered a biological structure that may be susceptible to electromagnetic radiation (1). This judgement is based on considerations of membrane structure as well as function. For example, the existence of large potential gradients due to asymmetrically charged macromolecular components, cell surface charge density and ion mobility, and interactions with structural water are believed to render membranes sensitive to oscillating electric and magnetic fields. Functional properties involving membrane dipolarization, active ion channels and gates, and transport processes involving charged compounds also contribute to the notion that membranes might be influenced by electromagnetic radiation. Membrane-associated bioeffects have been identified in excitable cells such as neurons and cardiac cells; however, these effects relate to the specialized electrical nature of the excitable cell membrane (2-5). A major unanswered question is whether such bioeffects exist for membranes of nonexcitable cells and, moreover, if effects are inducible, what specific conditions are required to elicit the response(s).

This question has been addressed using the erythrocyte in several in vitro bioeffects studies. These studies, surprisingly, have indicated that microwaves may alter or have no effect on cation permeability, the most frequently studied membrane parameter. These conflicting results suggest that as yet undefined factors mediate the permeability effects. Four research groups have reported altered cation permeability during microwave irradiation at room temperature (6-9). In contrast, three similar investigations have resulted in negative findings for microwave exposures performed at or near 37°C (10-12). The apparent role of exposure temperature recently suggested to us (13), and others (8,9) that a membrane phase

transition, believed to exist for erythrocytes at approximately 18-22oC (14), may be a possible basis for this permeability increase.

We have set out to test this hypothesis and, moreover, to identify factors that modulate microwave effects on cation permeability. Passive sodium permeability has been examined as a function of exposure temperature over the range of 13-43oC which includes the phase transition region of 18-22oC, T_c. The term phase transition is used here to identify a non-linear region of the Arrhenius plot reflecting a distinct change in activation energy for sodium transport. The results presented here demonstrate that the presence of a phase transition is important to microwave effects on membrane permeability. Pronounced increases in Na^+ permeability are observed only at T_c during microwave exposures and not for temperature-matched, sham exposures. Moreover, cholesterol modification of the erythrocyte cell membrane, which eliminates the phase transition at T_c, obliterates the microwave effect. This last fact establishes a link between the presence of a phase transition at T_c and microwave effects on cation permeability.

This microwave effect at T_c has also been investigated to identify factors that modulate this response. We report that plasma potentiates the microwave-induced increase in permeability and that oxygen also enhances the microwave effect. In contrast, the two antioxidants ascorbic acid and mercaptoethanol inhibit the microwave effect. Additional studies have shown that the permeability increase observed during microwave treatment is reversed post-exposure and slowly recovers over a 60 minute period. Taken together these findings raise important questions about the physical and chemical basis of the interaction between microwaves and the cell membrane at T_c.

EXPERIMENTAL PROCEDURES

Erythrocyte Preparation and Na-22 Uptake Measurements - Rabbit erythrocytes were prepared as described (15). Four male New Zealand rabbits of 8-10 months of age were bled at 30 day intervals from the marginal ear vein. The heparinized blood was washed in Ringer's buffer (pH 7.40) containing 11 $\underline{mM}$ glucose and 0.01% BSA. Care was taken to remove leukocytes so as to avoid the possibility of proteolytic activity; the top one-third of the packed cell suspension was discarded. Erythrocytes were judged morphologically normal and free of leukocyte contamination by light microscopy. Cells were used at 1×10^{10}/ml.

Cation permeability was assessed by quantitating Na-22 influx in ouabain-treated erythrocytes (16); efflux experiments were also performed using Na-22 equilibrium-loaded cells and this gave similar results, as expected. Briefly, cells were preconditioned in teflon test tubes to the appropriate pO_2 level by gentle bubbling (0.09 LPM) of premixed, prefiltered (0.45 μ) O_2/N_2 gas through the suspension for 15 minutes at a desired temperature. Changes in temperature and pO_2 did not alter pH levels, induce hemolysis, or affect cell morphology. Measurement of pO_2 was accomplished using a thermostatted Clark-type oxygen electrode before and after Na-22 assays on selected samples. After thermal and pO_2 equilibration was reached, microwave or sham samples were placed in the waveguide and 4 μCi of Na-22 (New England Nuclear, Boston, MA) per ml of cells were added, and at different times samples were removed and rapidly centrifuged (12,000 xg in 5 sec) at 4°C. The supernatant was discarded and the pellet resuspended in 1 ml of cold Ringers buffer containing 0.1 $\underline{mM}$ ouabain. This was repeated once and 100 λ of the packed erythrocytes were removed, carefully transferred to 100 λ of Ringers buffer, and the amount of Na-22 quantitated

using a gamma-well spectroscopy system employing an Ortec 7100 multichannel analyzer.

Preparation of Cholesterol Enriched Erythrocyte Membranes - Erythrocytes were enriched in membrane cholesterol by incubation in mixtures of sonicated cholesterol/phosphatidylcholine liposomes in the presence of albumin according to the methods of Cooper (17) and Deuticke (18). Control erythrocytes used in microwave and sham exposures were treated identically except that liposomes were absent from the incorporation protocol. No alterations in gross morphology of control or cholesterol-loaded cells were revealed by light microscopy. Cholesterol and lipid determinations of erythrocyte membrane were performed as previously described (19). The cholesterol/phospholipid (C/P) mole ratio increased from 0.5 to approximately 2.0. Since there was no change in the phospholipid content of the untreated or treated cells, this corresponds to an approximate four-fold increase in membrane cholesterol. This is in qualitative agreement with results obtained using human erythrocytes (17,19).

Microwave Apparatus and Exposure Protocol - Microwave and sham-exposures were conducted in a tunable waveguide device capable of continuous sample temperature measurement, continuous sample mixing, pO_2 conditioning, on-line forward and reflected power monitoring, and temperature control of the exposure compartment by a circulating, nonabsorbant dielectric fluid (15) (Figure 1). The waveguide was operated at 2,450 MHz (CW) in the TE_{10} mode, and was tuned to impedance match the sample and generator using two inductive-capacitative sliding elements and a sliding short to locate the peak standing wave 1/4 wavelength from the sample position. This enabled more than 99% of the incidence energy to be absorbed by the sample.

The erythrocyte suspension to be exposed was placed in a nonabsorbing, three-piece teflon exposure compartment depicted in Figure 2. The teflon sample cell (0.5 cm, OD) (A, Figure 2) was loaded with 1 ml of the erythrocyte suspension and placed into the teflon baffle insert which was positioned in the teflon waveguide insert (B,C; Figure 2). The teflon waveguide insert was placed in the center of the waveguide section with the sample cell parallel to the short dimension of the waveguide, and thus parallel to the electric field vector. This resulted in the sample being positioned in the middle third of the waveguide to insure uniform incident electric field intensity.

The sample cell had a silastic gas line (0.1 mm OD) and a VITEK thermistor probe (0.1 mm OD) (20), both nonabsorbing elements, located inside during both microwave and sham exposures. The capability for conditioning for pO_2 has not been previously incorporated into an in vitro exposure protocol; this enable simulation of physiological values of intracellular, venous, and arterial pO_2. In addition, since the premixed, prefiltered (0.45 µ) O_2/N_2 gas was gently bubbled at 0.09 LPM, the cell suspension was continuously mixed. This provided an effective means to eliminate thermal gradients during microwave exposures which in the past have lead to effects erroneously attributed to nonthermal microwave interactions (5,12).

During exposures the teflon sample cell was jacketed with a moving wall of dielectric fluid (6 L/min) to thermostat the sample at an equilibrium exposure temperature (+ 0.05oC). Dodecane or Dow Corning 200 were used since both are essentially nonabsorbing at 2,450 MHz (21). Power absorption could be varied at a constant sample temperature by adjusting the temperature of the circulating dielectric to compensate for the change

in absorbed power. An indirect means was also employed to monitor sample temperature during exposures. In this case, a Wheatstone bridge circuit, incorporating two matched A919a thermistors ($\pm$ 0.01°C, Thermometrics, Edison, NJ) measuring inflow and outflow temperatures of the circulating dielectric fluid, was used to continuously monitor the temperature differential between inflow and outflow thermistors. Sample temperature was correlated to this differential by comparing the latter to direct VITEK probe measurements. The A919a thermistors as well as the VITEK probe were each calibrated against an NBS-traceable S-10 temperature standard ($\pm$ 0.0015°C accuracy, Thermometrics, Edison, NJ).

<u>Measurement of Absorbed Power and Internal Electric Field</u> - The specific absorption rate (SAR) for microwave exposures of cell suspensions was determined from direct temperature measurements on nonthermostatted samples (5,12). The following equation was used to compute the SAR,

$$SAR(mW/g) = \frac{\Delta T \times C\rho}{1.16} \tag{1}$$

where ΔT is the temperature change in °C per hour, and $C\rho$ is the specific heat of the absorbing sample. A value of 0.83 kcal/°C kg for $C\rho$ was assumed for the erythrocyte suspension (22). Values of SAR obtained in this way were used to determine an equivalent internal electric field intensity present in the absorbing sample, which is distinct from the electric field incident on the sample, by using the relationship (23)

$$E_{in}(v/m) = \left[\frac{2\rho}{\sigma} SAR\right]^{\frac{1}{2}} \tag{2}$$

For the erythrocyte suspensions, a value of 1 kg/m^3 was used for ρ, and a

value of 2.0 mho/m at 2,450 MHz was used for σ (24).

Miscellaneous Methods and Reagents - Hematocrit values were deter-
mined using a direct reading centrifuge (Clay Adams MCHT II). Hemoglobin
determinations were accomplished using the cyanmethemoglobin method (Techn-
ical Bulletin, No. 525, Sigma Chemical Co., St. Louis, MO). All reagents
were research grade or better. Ascorbic acid and mercaptoethanol were pur-
chased from Sigma Chemical Co., St. Louis, MO. Double-distilled, deionized
water was used throughout.

RESULTS

Determination of Thermal Gradients in the Sample Cell: Effects of
Mixing - Since hot spots have been reported during in vitro exposures of
erythrocyte suspensions (9,12,25), we directly measured the temperature of
the sample volume along its length and width during irradiation. Figure 3
depicts the long-axis temperature profile of an erythrocyte sample for dif-
ferent values of SAR with and without mixing using atmospheric air (0.09
LPM). This family of curves corresponds to a steady-state sample tempera-
ture of 17°C at mid-height. These data indicate that in the absence of
mixing the bottom and the top of the sample volume displayed thermal gra-
dients, and that they became pronounced as SAR was increased. In contrast,
when mixing was employed these thermal gradients were significantly reduced
as detected by the VITEK probe. The maximum excursions observed were on
the order of 0.05°C at 650 mW/gm for a flow rate of 0.09 LPM. The effects
of varying SAR and of mixing shown here were qualitatively similar for
exposures at other sample temperatures.

An optimal bubble size and flow rate should exist that provides
effective mixing to eliminate thermal gradients while avoiding mechanical

stress to the cells. We employed 0.09 LPM since this was the lowest flow rate tested that yielded uniform sample temperature; hemolysis due to possible mechanical damage was not evident at 0.09 LPM or at higher flow rates of ∿0.25 LPM. Bubble diameter used was on the order of 0.5 mm (I.D. of silastic tubing) and at 0.09 LPM bubbles do not fuse to form larger structures. Power absorption discontinuities are not expected to become significant until air gaps of 1/4 wavelength are approached, i.e., cm or greater. Microscope examination revealed that erythrocytes in Ringer's buffer with 0.01% BSA fail to adhere to the surface of these bubbles, thus a moving bubble is expected to mix the cells without generating a small cell population maintained at an air-water interface.

Arrhenius Plot of Na-22 Influx During Microwave and Sham Exposures - To determine sodium influx, the uptake of Na-22 by ouabain-treated erythrocytes was measured at different times for temperatures in the range 13-43oC. Uptake was linear over a 60 minute period for microwave arɪ sr.m-treated cells and this agrees with previous reports for conventionally heated erythrocytes (16). The slopes of these lines were used to compute influx values in DPM/gm RBC/hr at various temperatures.

Arrhenius plots were constructed from these influx values and are depicted in Figure 4. Sham-exposed, normal erythrocytes revealed a single nonlinear region at 17.5-19.5oC, T_c, bounded by two linear regions extending to higher and lower temperatures. The difference in slopes of these two lines corresponded to an approximate doubling of activation energy as temperature was decreased (6.5 kcal/mole vs. 14 kcal/mole). This change in activation energy corresponds to that observed in previous reports (26). This nonlinearity is considered indicative of a protein and/or lipid phase transition within the cell membrane (27-28); erythrocytes from various

sources, including humans, are reported to exhibit this behavior within the range of 18-20oC (14).

Microwave exposure (100 mW/g) of normal erythrocytes at temperatures between 13-43oC revealed that only at T_c did Na-22 influx exceed that observed for sham-treated cells by 75-100%. Inspection reveals that a solvent temperature of approximately 28oC is required for the influx from sham-exposed cells to match that observed for microwave-treated cells exposed at 17.7oC. The small temperature fluctuations detected with the VITEK probe for the bulk solvent during exposures ($\leq$ 0.05oC) do not readily account for this 75-100% increase in permeability. In these experiments the question of bubbles interacting with the microwave field was addressed by substituting mechanical mixing using a teflon rod for gentle bubbling at atmospheric pO_2. During mechanical mixing sodium influx was also enhanced 75-100% at T_c for microwave compared to sham exposures. This indicates that mixing by gentle bubbling was not a confounding factor.

That the microwave-induced increase in permeability occurs at T_c suggests this effect may be linked to the presence of the cell membrane phase transition. To test this hypothesis, erythrocytes were modified so that their membrane cholesterol content was increased four-fold without altering the phospholipid content. Cholesterol-loaded erythrocytes are known to display reduced cation transport (18,19), and liposomes, when small amounts of cholesterol are incorporated, exhibit a broadened phase transition region while large amounts of cholesterol completely eliminate the phase transition (29). Figure 4 illustrates that sham-exposed, cholesterol-loaded erythrocytes displayed reduced Na-22 influx at all temperatures above 15oC without evidence of a membrane phase transition at T_c. The activation energy for these cells was observed to be identical to that

for normal erythrocytes maintained at temperatures above T_c. When cholesterol-loaded cells were examined for permeability effects during microwave exposures over the range 13-43°C no alterations were detected. These results link cation permeability effects in the erythrocyte to the presence of a phase transition at T_c.

Dependence of Microwave-Induced Permeability at T_c on Absorbed Power and the Internal Electric Field Strength - The dependence of the microwave-induced increase in Na-22 influx at T_c on SAR was characterized for exposures at 19.5°C. Figure 5 illustrates that the increase in Na-22 influx increased monotonically as SAR was increased and that a plateau was reached at approximately 400 mW/gm. In addition to Na-22 influx, the presence of hemoglobin (Hb) in the cell-free supernatant was quantitated; approximately 2% of total Hb available in the erythrocyte was released at the highest SAR employed. Total available Hb was taken as that released by osmotic lysis in cold distilled, deionized water. The 2% value for Hb release observed during microwave exposures was not significantly different from that observed for the sham-exposed cells.

The average internal electric field strength associated with the measured SAR, E_{IN}, was computed as per Equation 2. Figure 6 illustrates that a linear relationship was observed between E_{IN} and the microwave-induced increase in permeability. At an associated field strength of approximately 23 V/m this increase in permeability reached a maximum value.

Reversibility of Microwave-Induced Cation Permeability at T_c - To determine if the microwave effect is reversible, erythrocytes were exposed to microwaves (100 mW/g, 30 min) at 18°C and at staggered intervals thereafter Na-22 influx assays were performed. The results are depicted in Figure 7 and indicate that a reversal in Na-22 influx is observed

immediately following the termination of microwave exposure. This means that Na-22 influx was reduced compared to that for sham-exposed erythrocytes post-exposure. This reversal was found to be transient in that at 60 min following the termination of microwave exposure Na-22 influx was nearly identical to that for sham-exposed cells.

Dependence of Microwave-Induced Cation Permeability at T_c on Oxygen Tension - To simulate in vivo values of oxygen tension during microwave and sham exposures the atmospheric air mixture employed to maintain erythrocytes in suspension was replaced with premixed, prefiltered combinations of O_2 and N_2 gas. Values of intracellular (0-5 mm Hg), venous (35 mm Hg), arterial (90 mm Hg), atmospheric (155 mm Hg), and hyperoxic (760 mm Hg) pO_2 were simulated and the dose-response relationship for microwave (60 mW/gm) and sham treatment at $T_c = 17.7^oC$ is shown in Figure 8. Variation in pO_2 had no observable effect on Na-22 influx in sham-exposed cells. In contrast, a U-shaped dose-response curve was observed for microwave-exposed cells. Thus, the increase in Na-22 influx observed for microwave-exposed erythrocytes at atmospheric pO_2 (155 mm Hg) (Figure 4) was further enhanced at relative hypoxia (0-5 mm Hg) and hyperoxia (760 mm Hg). These results indicate that oxygen or a reactive oxygen species plays a role in modulating the permeability increase at T_c.

Dependence of Microwave-Induced Cation Permeability at T_c on Plasma and on Antioxidants - To determine the effect of naturally occurring extracellular factors such as proteins and lipids on the microwave permeability increase at T_c exposures were conducted using erythrocytes suspended in mixtures of Ringer's buffer and normal rabbit plasma. The data in Table I reveal two effects due to plasma. First, the presence of plasma was observed to markedly enhance Na-22 influx for both sham (8,175 vs 6,365

DPM/gm RBC/hr x 10-5) and microwave (28,196 vs. 11,101 DPM/gm RBC/hr x 10-5) exposed cells. Second, when microwave and sham groups are compared, the presence of plasma is seen to be associated with a significantly greater increase in Na-22 influx due to microwaves (244% and 193% vs 74%). These findings indicate that an extracellular plasma component(s) increases passive diffusion of Na^+ and also significantly potentiates the microwave-induced permeability increase observed at T_c.

In addition to plasma, the effects of extracellular antioxidants were investigated since the strong pO_2 dependence suggested that oxygen or a reactive oxygen species participates in the reaction. Table I shows that when 5 mM mercaptoethanol or 5 mM ascorbic acid is present during exposures at atmospheric pO_2 the microwave-induced increase in cation permeability is not detected.

DISCUSSION

There exists at present no clear guidelines identifying conditions that influence microwave bioeffects on the cell membrane, except, perhaps, that high SAR can result in frank hyperthemia and elicit thermally-related responses. The results presented here demonstrate that microwave effects on erythrocyte permeability are restricted to a narrow temperature range and linked to the presence of a membrane phase transition. Moreover, these results reveal that this microwave effect is modulated by an extracellular factor(s) in blood plasma, in addition to oxygen and antioxidants. Taken together these findings provide insight into the physical and chemical nature of the interaction operating between microwaves and the cell membrane. Specifically, these findings help to characterize how three physiologically important factors, temperature, plasma, and oxygen, affect mem-

brane responses to nonionizing radiation.

During these experiments the VITEK probe did not detect sample temperature excursions more than $\pm$ 0.05oC over 0-400 mW/g. Since the 1/e response time of the VITEK probe is on the order of milliseconds, however, it is possible that larger excursions recovering within milliseconds may have occurred. Within this limitation, the microwave exposed solvent phase is assumed to be maintained at the equilibrium temperatures reported. Sham exposed samples were carefully temperature-matched to the microwave samples in all of these experiments; this permitted effects strictly due to solvent temperature to be identified and compared to that observed during microwave treatment. The above procedures rule-out the possibility that long-lived, gross thermal differences exist between the bulk solvent phase of the microwave and sham samples. Therefore, the microwave-associated effects we report most likely reflect differences in energy deposition and distribution within the cell membrane, at its adjacent surface or inside the cell interior, which occur at equivalent bulk solvent temperatures. For example, a simple thermal-based explanation for these results might involve micro-hotspots at the cell membrane, perhaps near the sodium channel. Although direct evidence for micro-hotspots in cell membranes has not been reported, unique structural changes do occur at T_c, as discussed below, that may bear on this question.

The striking temperature dependence we observe perhaps provides the most insight regarding membrane susceptibility to microwaves (Figure 4). The microwave effect at ~17.7-19.5oC coincides with a nonlinearity in the Arrhenius plot which reflects the presence of a membrane phase transition (14,27,28). Moreover, we observe that cholesterol-loading which completely abolishes the phase transition obliterates the microwave effect, and this

demonstrates a link between the presence of the phase transition and microwave susceptibility.

How might the presence of a phase transition render the cell membrane susceptible to microwaves? The cell membrane is known to exhibit protein-phospholipid clusters that coexist with free phospholipid at T_c; importantly, at T_c, these clusters extrude protein from the cell surface (30). The extrusion of proteins is expected to alter the cell surface through spatial variations in polar amino acid side chains of proteins and associated bound water. Both of these classes of molecular dipoles are strong absorbers of 2,450 MHz (31). As a result, at T_c, microwave exposure could result in a pattern of energy transfer quite distinct from that operating at other temperatures. In addition, the protein-lipid clusters that occur at T_c most likely represent regions with different bulk dielectric properties; this could also contribute to unique absorption characteristics at T_c. Regardless of these structural considerations, the demonstration that cholesterol-loaded cell membranes, which do not exhibit a phase transition at T_c, fail to respond to microwaves indicates that the membrane phase transition plays a major role in an interaction mechanism.

An Arrhenius analysis has been used in only two previous studies of cation permeability in erythrocytes (7,8). Olcerst et al. reported an increase in passive Na-22 efflux from rabbit erythrocytes during 2,450 MHz exposures at 12, 22.5, and 36ºC where their Arrhenius plot exhibited three vertical "zig-zags". These temperatures, however, were not associated with a membrane phase transition since the slope, and thus activation energy, above and below each "zig-zag" was unchanged. Such a zig-zag feature has not previously been reported and its significance is not readily interpretable. We were unable to reproduce this feature using the same apparatus

and protocols. In the study reported here we maintained cells in suspension and directly measured sample temperature during exposures. Factors relating to erythrocyte settling, which is significant at 30 min, thermal hot spots, and the lack of direct temperature measurements may account for the results of this earlier study. Fisher reported an increase in passive sodium permeability for human erythrocytes at 22-25oC during 2,450 MHz exposures over the range 7-35oC. This suggests that effects on permeability in human red cells are also temperature dependent. Interestingly, an effect at 22-25oC suggests phase transition involvement, however, cholesterol-loaded cells were not employed to test whether a phase transition played a role in the effect.

The microwave effect at T_c depends on SAR (Figure 5) and E_{IN} (Figure 6), and it is significant that both dose-response relationships extrapolate to the origin for zero effect. Thus, a low-dose threshold or "trigger" value was not revealed in this study. This suggests that a zero effect may only be associated with a vanishingly small SAR or E_{IN}. Long-term, low-level exposures will need to be performed to confirm this possibility. In contrast, at high values of SAR and E_{IN} a limit for the permeability increase is detected.

We observed that the microwave-induced increase in Na-22 permeability is reversible and that this "rebound" effect is transient (Figure 7). A simple explanation for this response is that the effective size of the sodium "pore" or channel might be enlarged during microwave exposure; this would involve a reversible change in steric hinderance. Alternatively, microwaves could increase the efficiency of the carrier protein believed to mediate Na/K cotransport (32); this is also a physical effect but it would involve a catalytic-like structural change that improves protein function

during microwave exposures. Until more is known about the molecular nature of passive transport a distinction between these or other possible modes of interaction cannot be made. The transient "rebound" in Na influx we detect cannot be attributed to a build-up of sodium inside the cell, since both influx and efflux are increased identically during microwave exposure (data not shown); such a build-up of sodium would lead to observable changes in cell volume and morphology which we did not detect. Cleary and colleagues have recently observed that microwave-induced increases in K efflux of ouabain-untreated rabbit erythrocytes exposed in whole blood at 22°C are reversed at 1 hour post exposure (33); they attribute this effect to the Na-K pump correcting the cation imbalance. Thus, it would appear that microwave effects on both active and passive transport in the rabbit erythrocyte are reversible.

The strong pO_2 dependence observed in this study (Figure 8) indicates that oxygen modulates the microwave effect. The major binding site for molecular oxygen in the erythrocyte is Hb, and a small fraction of Hb is reported to bind to the cell membrane with Band 3 protein (34). In addition, at T_c, the vertical diffusion of oxygen across model phospholipid membranes has been shown to increase abruptly (35). These findings suggest the possibility that at T_c more oxygen may move across the erythrocyte and be available to membrane-bound Hb. When oxygen binds to Hb in solution, its protein conformation is altered and this is reported to induce a reversible increase in the dielectric increment when measured at megahertz frequencies (36). Whether such an effect operates at 2,450 MHz and for membrane-bound Hb is unknown, however, such a situation might render the membrane unique with regard to absorption of microwave energy and result in an effect on membrane permeability. Studies using erythrocyte ghosts should enable a

determination of whether Hb plays a role in the oxygen dependence reported here.

In addition to oxygen, blood plasma also modulates the microwave effect (Table I). The observation that plasma itself enhances Na-22 permeability during conventional heating compared to that for Ringer's buffer indicates that a plasma component(s) interacts with the membrane under physiological conditions. This plasma effect on passive cation diffusion has not been previously reported and a specific plasma component(s) and its site of interaction is as yet undetermined. In contrast, many studies have identified effects on active and passive cation transport in the erythrocyte induced by alterations in in situ membrane cholesterol and phospholipid content (37). Likely extracellular factors to consider as potential candidates mediating the plasma effect we observe are lipoproteins. Plasma apolipoproteins, in particular, have been shown to spontaneously insert into liposomal membranes (38); interestingly, the rate of penetration is maximal at the phase transition temperature. Such an insertion process might be influenced by microwaves and affect cation permeability. Our observation that plasma leads to a striking increase in the microwave effect indicates that a physiological blood component(s) mediates the erythrocyte response to microwaves. Future studies using individual plasma components, such as proteins, lipoproteins, and apolipoproteins, should help to identify a specific factor(s) involved in this potentiation.

The observation that oxygen potentiates the microwave increase in permeability (Figure 8) and that the presence of two antioxidants inhibit the microwave effect (Table I) further suggests that modulation of this interaction has a chemical basis. Both antioxidants used are free radical scavengers (39,40) and they may participate by neutralizing a reactive

oxygen species during irradiation. Free radicals can initiate lipid perox-idation which can increase membrane permeability (41). If free radical species are involved it is energetically improbable that microwaves parti-cipate directly in their generation since the ionization potential for hydrogen is 12 eV and a single photon of 2,450 MHz radiation is equivalent to $\sim 10^{-4}$ eV (42). Alternatively, it is more likely that microwaves might lead to an accumulation of free radical species by inhibiting anti-autoxidation pathways that exist in the erythrocyte (43). Autoxidation is controlled inside the erythrocyte by the action of superoxide dimustase (SOD) and catalase, which eliminate O_2, and H_2O_2 generated by SOD, respec-tively. Autoxidation of oxyhemoglobin to methemoglobin occurs at a rate of about 3% per day and if either enzyme is impaired during microwave expo-sure, O_2 or H2O2 could accumulate and this might result in membrane damage. The presence of ascorbic acid or mercaptoethanol acting as antioxidants would be expected to diminish this effect, as we have observed.

ACKNOWLEDGEMENTS

TABLE I

Effects of Plasma and Antioxidants on the

Microwave-Induced Increase in Na-22 Permeability at T_c (17.7°C)

Agent[a]	Na-22 Influx[b]		Percent Change (MW-Sham/Sham)
	MW	Sham	
Ringers	11,101 ± 150	6,365 ± 153	74%
Ringers + Plasma (1:1 v/v)	28,196 ± 240	8,175 ± 194	244%
Ringers + Plasma (2:1 v/v)	21,975 ± 203	7,500 ± 131	193%
Ringers + 5 mM Ascorbic Acid	6,380 ± 193	6,511 ± 180	---
Ringers + 5 mM Mercaptoethanol	7,272 ± 205	6,889 ± 201	---

[a] Erythrocytes at 10^{10} cells/ml in Ringers (pH 7.4, 17.7°C),

0.01% BSA. Exposures at 60 mW/g, 30 min, 17.7°C, atmospheric pO_2.

Mean ± SD, n = 6.

[b] DPM/gm RBC/hr x 10^{-5}.

FIGURE LEGENDS

Figure 1: <u>In vitro</u> microwave apparatus. Device enables continuous sample temperature monitoring and control, pO_2 conditioning, and sample mixing. Tuning to minimize reflected power results in absorption of 99% of the forward power.

Figure 2: Teflon exposure assembly. A baffle directs a low dielectric coolant, such as Dow Corning 200, around the sample cell. Teflon and Dow 200 are transparent to microwaves.

Figure 3: Effect of mixing on sample exposure temperature. Mixing was achieved by gentle bubbling with prefiltered, atmospheric air (0.09 LPM). Erythrocytes at 10^{10} cells/ml, Ringer's buffer, pH 7.4.

Figure 4: Arhennius plot of sodium influx for microwave-exposed, normal and cholesterol-enriched erythrocytes. Microwave treatment at 100 mW/g, 30 min, atmospheric pO_2. Sham exposures were performed at matched sample temperatures in the unenergized waveguide, 30 min, atmospheric pO_2. Erythrocytes at 10^{10} cells/ml, Ringer's buffer, pH 7.4.

Figure 5: Power absorption dose-response. Specific absorbed power rate (SAR) for exposures at 19.5°C (T_c), 30 min, atmospheric pO_2. Hemoglobin (Hb) release expressed as percent of total available Hb/cell. Erythrocytes as in Figure 4. Mean $\pm$ SD, n=5.

Figure 6: Internal electric field (E_{IN}) dose-response. E_{IN} was computed from direct SAR measurements (refer to text and Figure 5), and corresponds to an associated electric field within the erythrocyte sample.

Figure 7: Reversibility and recovery of microwave-induced permeability

increase. Microwave exposures were conducted at 100 mW/gm, 30 min, atmospheric pO_2, 18ºC (T_c). Erythrocytes as in Figure 4. Mean $\pm$ SD, n=5.

Figure 8: Oxygen dose-response. Microwave exposures conducted at 60 mW/gm, 30 min, 17.7ºC (T_c). Erythrocytes continuously conditioned for pO_2 by O_2/N_2 gas bubbling (0.09 LPM). Sham exposures at matched temperature and pO_2. Erythrocytes at 10^{10} cells/ml, Ringer's buffer, pH 7.4.
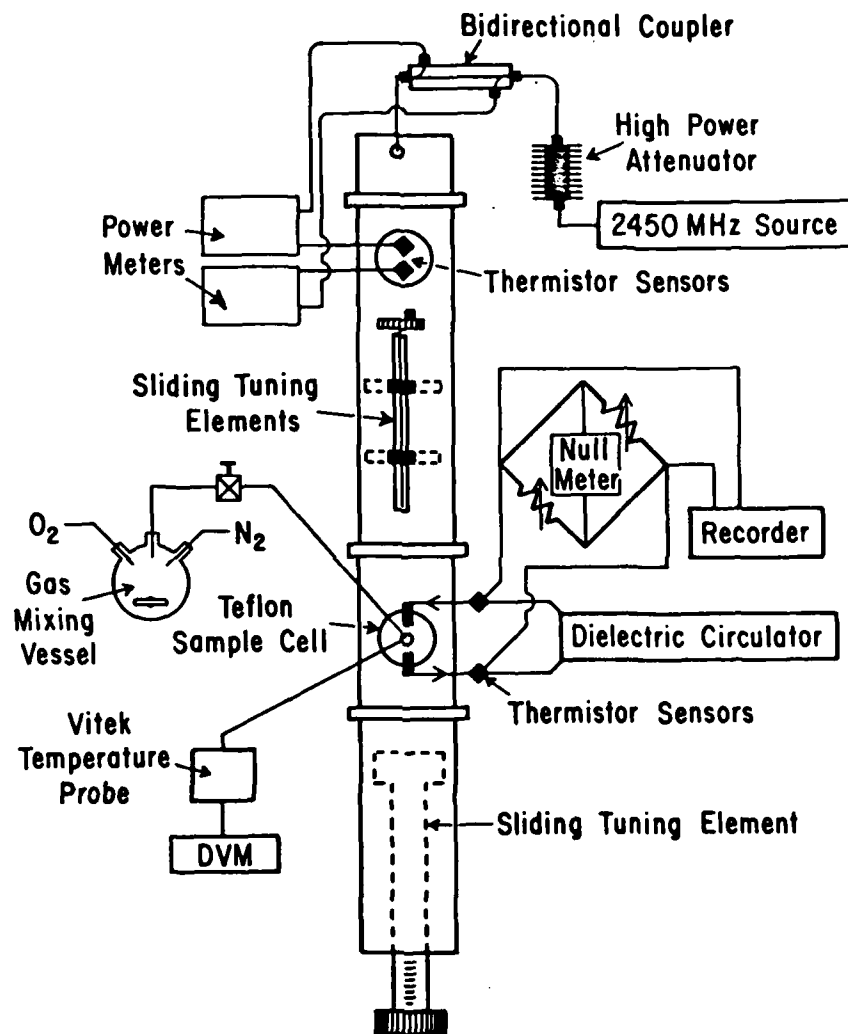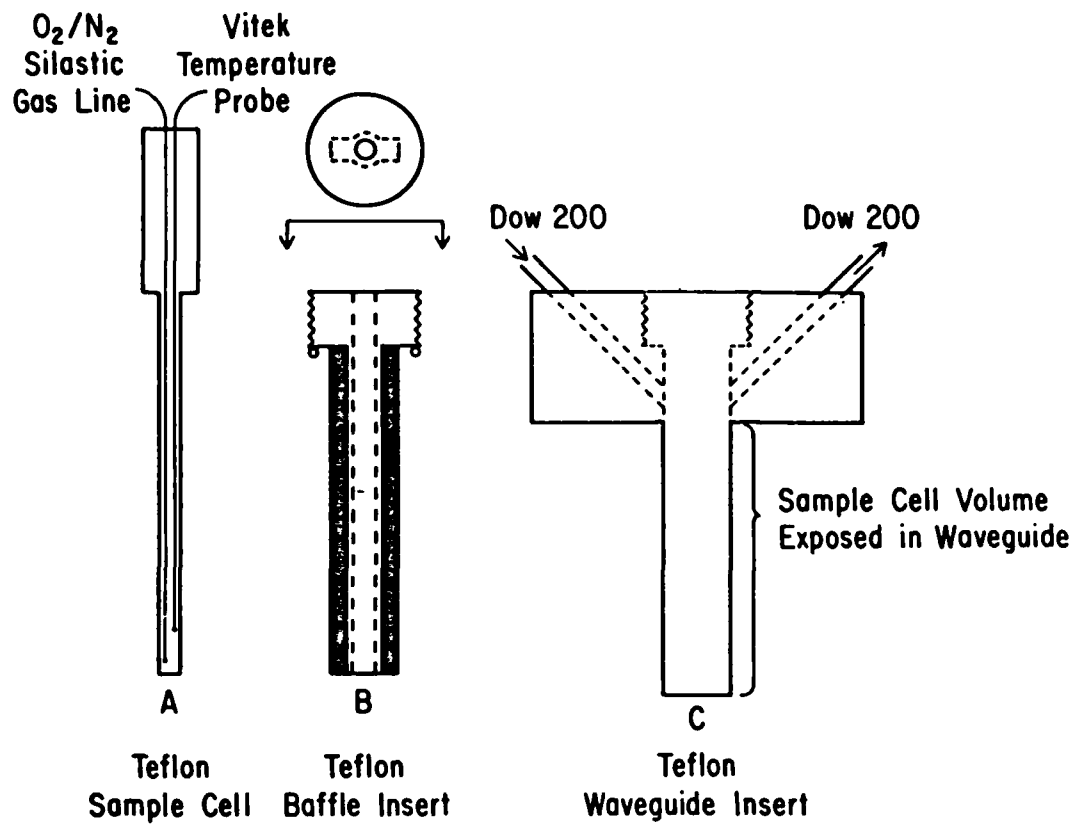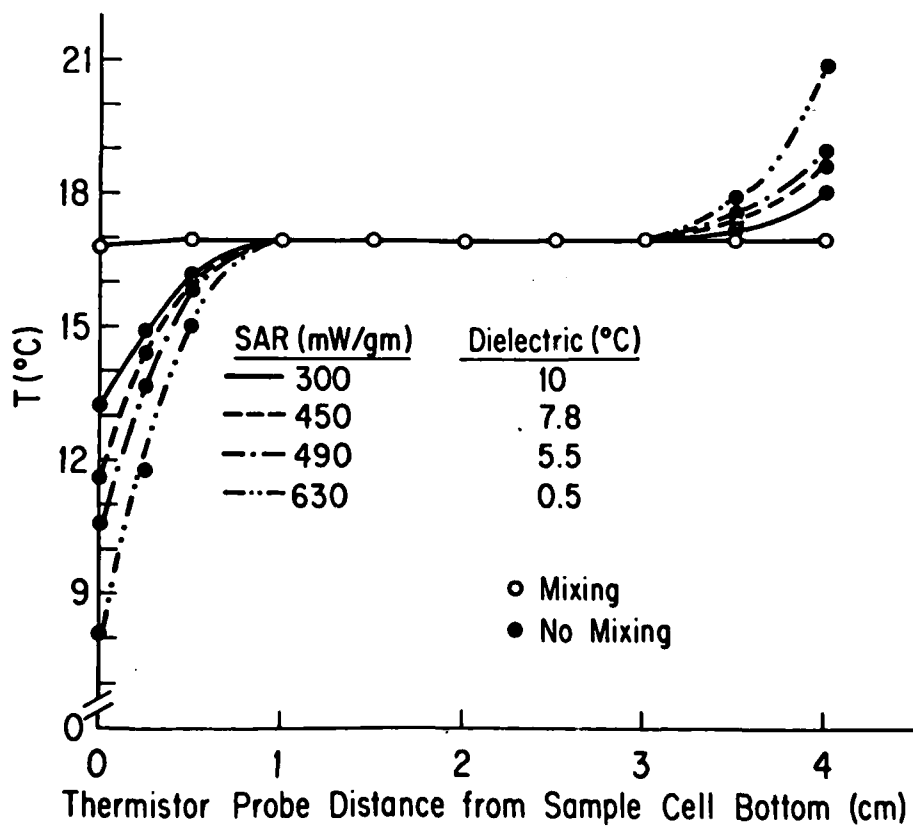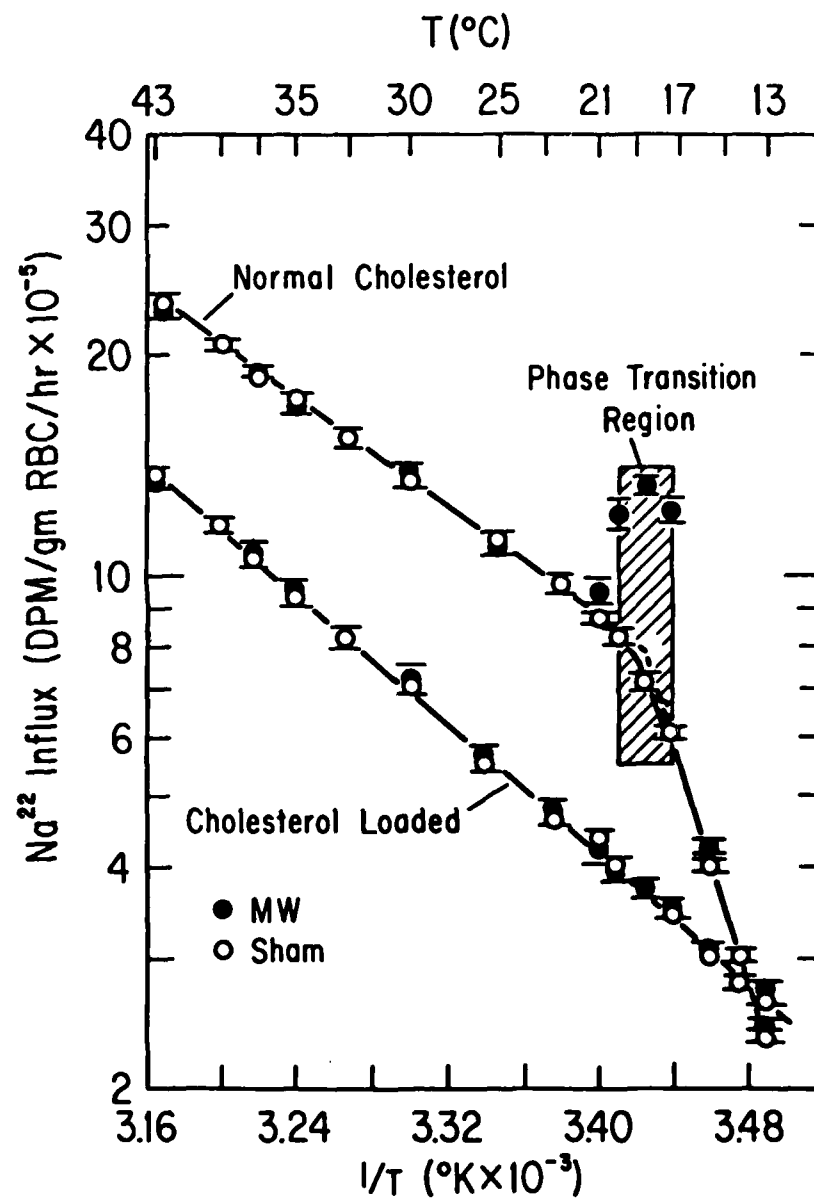
FIGURE 1

FIGURE 2   Liburdy and Vanek - 26

FIGURE 3

Thermistor Probe Distance from Sample Cell Bottom (cm)

FIGURE 4

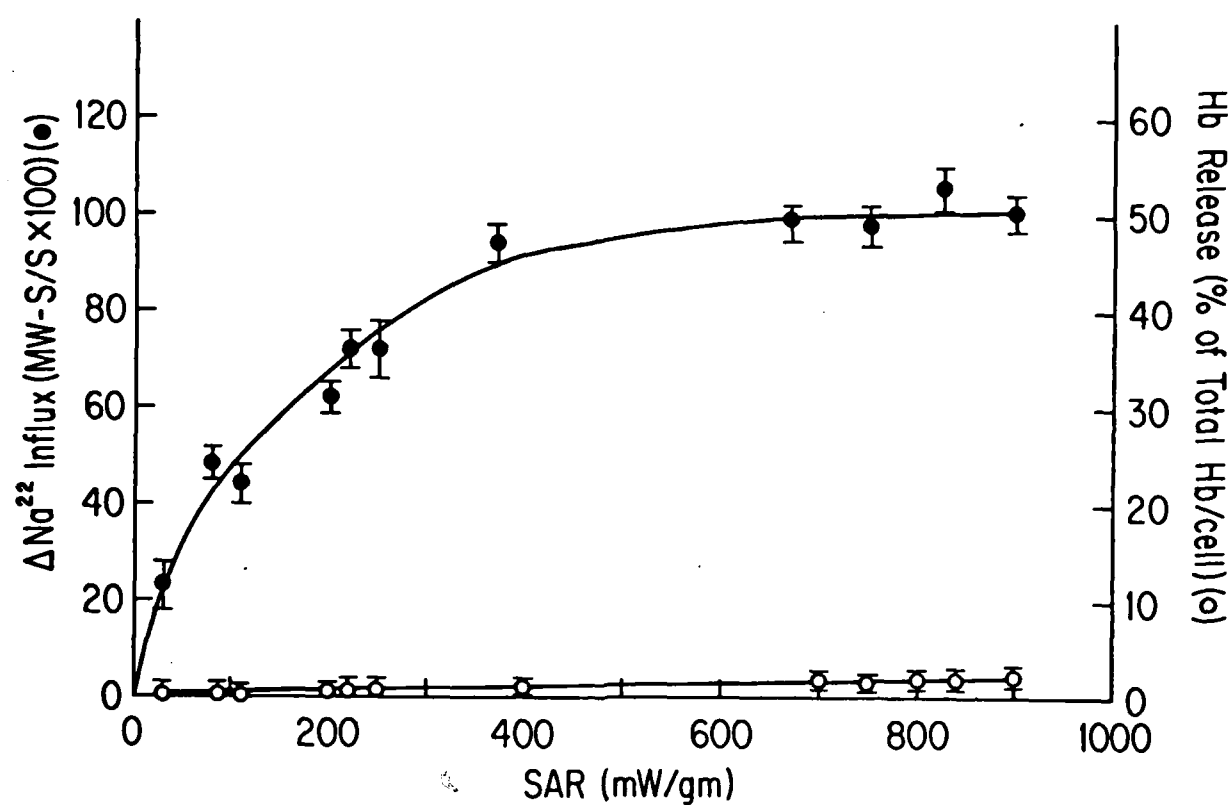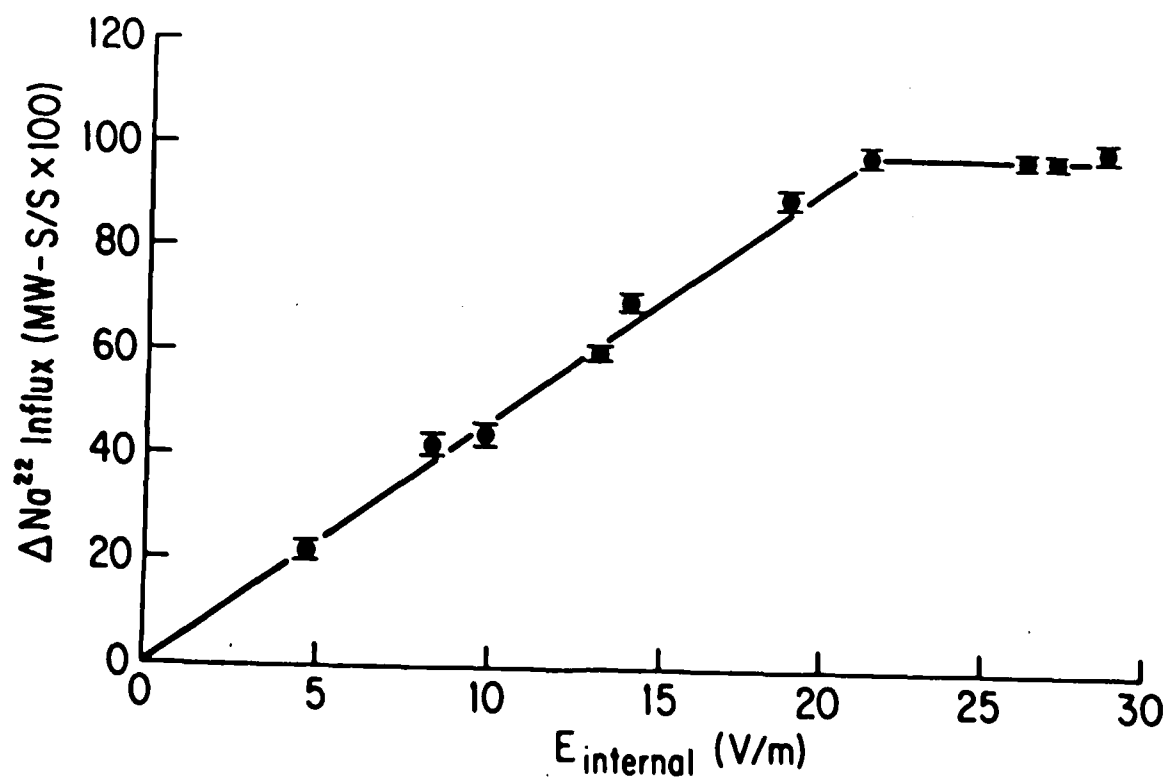FIGURE 5 Liburdy and Vanek – 28
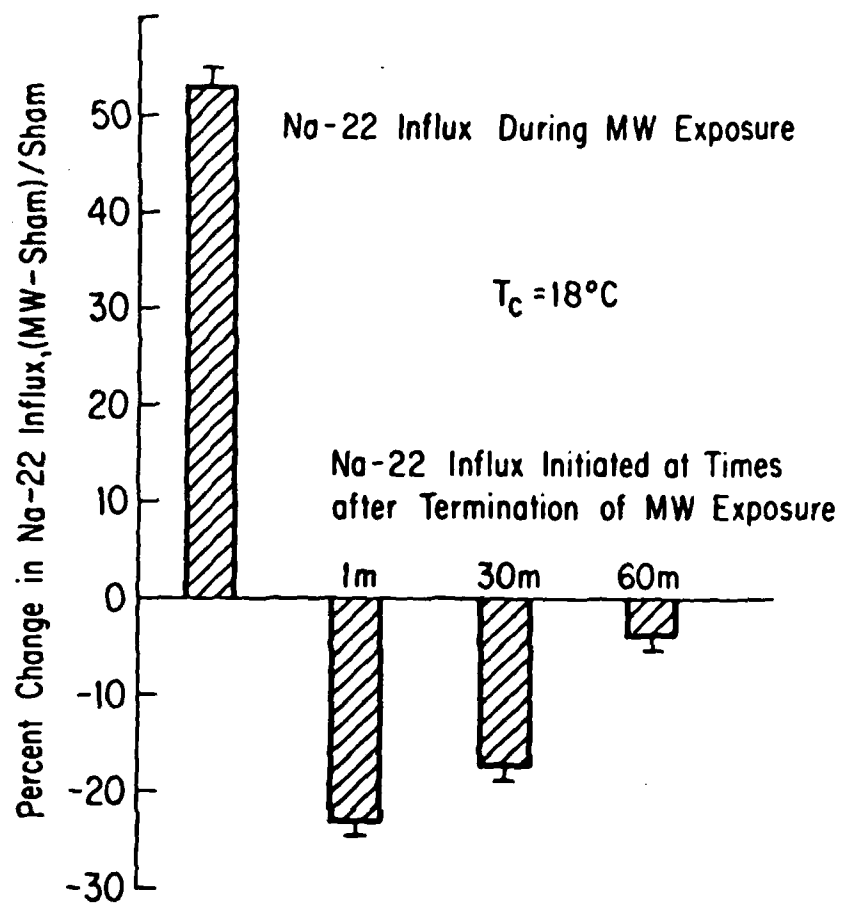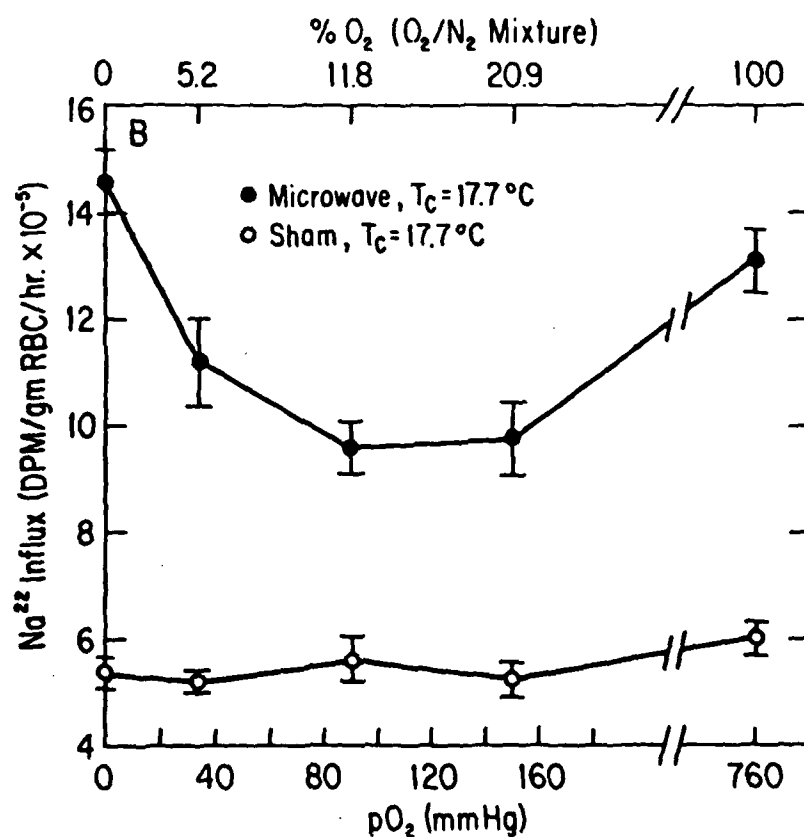

FIGURE 5

FIGURE 6

FIGURE 7

FIGURE 8

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MICROWAVE STIMULATED

DRUG RELEASE FROM LIPOSOMES

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ABSTRACT

Microwaves (2,450 MHz) are shown to stimulate the rapid release of a chemotherapeutic drug from liposome vesicles. This effect occurs at temperatures below the membrane phase transition temperature of 41.4oC where these liposomes are normally not leaky. In buffered saline a two second exposure (60 mW/g) triggers the onset of drug release at 31oC, whereas in plasma a near maximal release is observed as low as 25oC. This drug release is a linear function of microwave power and can be enhanced by oxygen and attenuated by antioxidants. These results suggest a means for relatively rapid and localized drug delivery in vivo and indicate that liposomes may provide a useful model for studying the interaction mechanisms between nonionizing electromagnetic radiation and biological membranes.

One approach to selective drug delivery to tumors is to employ liposome carriers that release chemotherapeutic agents when the temperature is raised above the solid-to-liquid phase transition temperature, T_c, of the membrane phospholipids (1). Microwave energy (2,450 MHz) has been used in such studies to achieve drug release during hyperthermia and this suggests the possibility of an interaction between microwaves, heat, and liposomes near T_c. Other evidence that microwaves may influence transmembrane movement near T_c was found using erythrocytes (2). When erythrocytes were exposed to 2,450 MHz microwaves they

experienced a two-fold increase in cation permeability, but only when irradiated at temperatures within the phase transition region of 17-19°C. An analogous increase in permeability during microwave exposures of liposomes at their T_c would facilitate drug release and, since microwaves can be focused in tissue within the constraints of wavelength and penetration depth (3), spatial selectivity would also be improved. We report that microwave energy stimulates a rapid and near maximal drug release from liposomes at T_c (41.4°C) and, more importantly, that drug release also occurs at exposure temperatures as low as 25°C, where the liposomes are normally not leaky. This last fact suggests that microwaves may enable rapid, spatially localized drug delivery via liposomes without the need to employ hyperthermia to reach T_c, and raises questions about the nature of biological membrane interactions with microwave radiation.

In these experiments microwave exposures were conducted in a tunable waveguide device operating at 2,450 MHz (Figure 1a). A 0.5 ml volume of liposomes was placed in a nonabsorbing teflon sample cell that was thermojacketed with a moving layer of nonabsorbing Dow Corning 200 (Figure 2b). Liposomes were positioned in the waveguide parallel to the E-field and sample temperature was monitored continuously using a nonperturbing Vitek thermistor probe ($\pm$ 0.01°C) (4). Prefiltered gas was gently bubbled at 0.09 L/min to condition the liposome suspension for oxygen and nitrogen and also to mix the liposomes. This arrangement resulted in uniform sample temperature ($\pm$ 0.05°C) throughout the suspension

for exposures conducted at 60 mW/g specific absorption rate (SAR) (5).

Liposome vesicles containing tritiated cytosine arabino-furanoside ([3H]-ARA-C, m.w. 243) were prepared using the reverse phase evaporation process from two highly purified and completely saturated phospholipids, dipalmitylphosphatidylcholine (DPPC) and dipalmitylphosphatidylglycerol (6). These liposomes are believed to be predominantly large unilamellar vesicles (6). Release of [3H]-ARA-C from liposomes as a function of temperature is shown in Figure 2a. Sham-exposures of liposomes in buffered saline for ten minutes resulted in a 60% maximal release at temperatures of 39.5^{o}C and above. This abrupt change in permeability occurs slightly below the T_c of 41.4^{o}C for these lipids (7). In contrast, microwaves triggered the onset of drug release at 31.5^{o}C with 58% maximal release achieved at 38°C. No drug release was detected at these temperatures in the absence of microwaves. When microwave power was varied from 3 to 60 mW/g at 38°C drug release increased in a linear fashion (data not shown). The 58% maximal release at high power occurred within seconds, however a 58% maximal release could also be achieved by using lower rates of absorbed power and longer exposure times. Although no difference in drug release was observed between mechanical (teflon rod) or gas mixing at atmospheric pO_2, we also conducted exposures at low levels of absorbed power (3 mW/g) without any mixing to rule out possible effects due to interactions with bubbles. A small sample volume of 0.1 ml was used to minimize nonuniform power

absorption in the absence of mixing, and for 3 mW/g temperature differences were $\geq$ 0.06°C within the sample volume. Microwave stimulated drug release at 33 $\pm$ 0.06°C (30 min) without bubbling was identical to that for mixing by gentle bubbling at atmospheric pO_2. Thus, gentle bubbling does not appear to be a confounding factor.

To determine whether microwave-stimulated drug release is influenced by the presence of plasma proteins and phospholipids, exposures were conducted using liposomes suspended in equal volumes of buffered saline and rabbit plasma. Figure 2a reveals that in the presence of plasma, sham-exposed liposomes release 80% of their contents at 39.5°C. Plasma itself, therefore, enhanced drug release at T_c. When microwave exposures were conducted an 80% release was also observed at T_c, but the presence of blood plasma resulted in 80% release at all lower temperatures studied. At 25°C this drug release was complete after a two second exposure (data not shown).

Since pO_2 varies considerably in vivo, the effect of oxygen and nitrogen on the liposome response to microwaves was also characterized. Figure 2b depicts heat release curves for liposomes in the presence of O_2, N_2, and atmospheric air. Sham-exposed liposomes displayed a normal change in permeability at 39°C without exhibiting oxygen dependence. Microwave exposures, however, resulted in enhanced drug release in the presence of O_2 with a significant broadening of the heat release curve. Interestingly, this effect was only observed for oxygen while atmospheric air and nitrogen gave identical results. Although

these liposomes were made from completely saturated phospholipids and, thus, possess no oxidizable acyl carbons, the above results indicate that oxygen does play a role in this permeability change.

To determine whether antioxidants influence microwave stimulated drug relase, liposomes were suspended in buffered saline and exposed in the presence of ascorbic acid and mercaptoethanol. Replacing O_2 with N_2 and conducting exposures in the presence of ascorbic acid or mercaptoethanol reduced microwave-stimulated drug release (Table 1). Thin-layer chromatography of the liposome suspensions after microwave treatment revealed no evidence of lipid degradation (8).

The exact nature of the physical and/or chemical interaction(s) responsible for microwave stimulated drug release is not yet known. Gross thermal effects due to unequal solvent heating are unlikely since temperature was carefully controlled and monitored during microwave exposures, and since sham-exposed, temperature-matched samples were used for comparison. This does not, however, rule out the possibility of microthermal effects at the liposome surface, within the liposome membrane or inside the vesicles.

Microwaves have been reported to have no effect on the lateral diffusion of lipophilic dyes within multilamellar phospholipid vesicles (9), and within erythrocyte membrane bilayers (10), as judged by steady-state fluorescence depolarization measurements during microwave exposures. Microwaves are also reported to have no effect on cation diffusion across small unilamellar

vesicles (11); however, this negative permeability result was obtained during exposures conducted far from the phase transition temperature of -7oC (12), in the absence of serum or plasma, and with no O_2 conditioning. In contrast, microwaves have been reported to increase passive cation diffusion through erythrocyte membranes for exposures at the membrane phase transition tempera- ture (2,13). This effect is strongly dependent on pO_2 (2) and is eliminated when the membrane is enriched with cholesterol to obliterate the phase transition (14). The results presented here using liposomes which are devoid of proteins suggest that phos- pholipids are an important target for microwave radiation. More- over, the strong dependence of drug release on O_2, its potentia- tion by plasma, and its inhibition by antioxidants, suggest a chemical basis of interaction, and indicate that liposomes should be useful models for the study of biological membrane responses to microwaves (15).

Microwave stimulated drug release from liposomes has the potential to significantly improve localized drug delivery in vivo. Microwaves are currently used to achieve localized heating in vivo with devices such as direct contact and phased-array applicators (16); these could be used to stimulate rapid, local- ized drug release from target areas containing circulating lipo- somes. The liposomes we used were designed to circulate in vivo without releasing their contents unless heated by conventional means to $\geq$ 39.5oC (17). Since microwave exposure of these lipo- somes in the presence of plasma at temperatures as low as 25oC results in 80% maximal drug release, the need for hyperthermia

and for elaborate in vivo temperature monitoring is not necessary. As a result the adverse side effects due to tissue heating, would be eliminated. It may also be possible to take advantage of the potentiating effect of O_2 on drug release. For example, by hyperoxygenating target areas, drug release might be enhanced, and administering antioxidants to nontarget sites might reduce drug delivery in these areas. Clearly, the use of microwaves and liposomes in this way represents a new approach to drug delivery, however, a number factors such as exposure duration and kinetics need to be investigated in vivo before clinically effective strategies can be formulated.

FIGURE LEGENDS

Figure 1:   Microwave Exposure Apparatus and Sample Cell.

(1a) Microwave exposures were conducted for 10 min at 60 mW/g in a tunable rectangular (5.5 x 11 cm) waveguide device operating at 2,450 MHz (CW) in the TE_{10} mode. Forward and reverse power were monitored and greater than 99% of the forward power was absorbed by the sample when the tuning elements were properly positioned. Liposomes were exposed in a teflon sample cell placed inside the waveguide parallel to the E-field (short axis direction) with the sample volume in the middle third of the waveguide to insure uniform exposure. Sham-exposures were conducted in an unenergized waveguide; sample temperature in this case was maintained by circulating Dow Corning 200, a silicone fluid, around the sample cell as described below. Both teflon and Dow 200 are relatively transparent to 2,450 MHz radiation (18).

(1b) A teflon sample cell (6 mm, I.D.) was filled with approximately 0.5 ml of liposome suspension and placed in a teflon baffle and a teflon waveguide insert. Dow Corning 200 was circulated at 6 1/min around the sample cell to facilitate heat transfer and this enabled microwave exposures to be conducted at different sample temperatures while maintaining a constant specific absorbed dose rate (SAR) (5). To

increase or decrease sample temperature the temperature of the Dow 200 was adjusted up or down, respectively, and the temperature off-set between sample and Dow 200 was found to be a linear function of SAR. For example, 60 mW/g ($0.86^{\circ}C$/min heating rate) required Dow 200 to be circulated at $^{\sim}5^{\circ}C$ cooler than the steady-state sample temperature desired during irradiation. Prefiltered (0.45 μm) gas at 0.09 l/min was delivered via a silastic tube (1 mm O.D.) to mix the liposome suspension during irradiation and to condition the solution with oxygen or nitrogen. This mixing action eliminated local hot spots as determined by a Vitek temperature probe (1 mm O.D.). This probe is nonperturbing to microwave fields and was used to measure sample temperature ($\pm0.01^{\circ}C$) continuously during exposures (4). Maximum excursion in steady-state sample temperature detected for exposures at 60 mW/g with bubbling and circulating coolant was $0.05^{\circ}C$ throughout the length and width of the sample volume. Quantitatively similar temperature excursions were observed using a 0.3 mm teflon rod to mix the suspension.

Figure 2:   Microwave Stimulated Drug Release from Liposomes. (2a) Drug release curves for liposomes heated by microwaves (60 mW/g, 10 min) and by conventional means; effects in buffered saline and plasma. Liposome vesicles were prepared by reverse phase

evaporation using a 1:4 (w/w) ratio of dipalmityl-phosphatidylcholine (DPPC) to dipalmitylphosphatidyl-glycerol (DPPG) obtained from Avanti Biochemical Co. (Birmingham, AL). Tritiated cytosine arabinofurano-side ($[^3H]$-ARA-C) was encapsulated into the aqueous compartment and $[^{14}C]$-DPPC into the lipid membrane; both were obtained from Amersham Corp. (Arlington Hts, IL). Liposome suspensions were filtered through 0.45 μm cellulose membranes and diluted to a working concentration of 3.1 mg/ml lipid. Drug release was assessed by measuring the radioactivity in superna-tants of ultracentrifuged (180,000 xg) liposomes. The low level of $[^{14}C]$-DPPC activity detected indicates that the liposomes were completely separated from the supernatants. Maximal drug release was induced by lysing liposomes in liquid scintillation cocktail. During exposures liposomes were suspended in normal saline with 25 mM Hepes (pH 7.4), or in equal volumes of buffered saline and rabbit plasma. Atmospheric air was filtered through cellulose (0.45 μ pore size) and bubbled at 0.90 l/min into the liposome suspen-sion.

(2b) Drug release curves for liposomes as a function of oxygen tension. Liposomes were exposed in buf-fered saline and assayed for drug release as described above with the exception that prefiltered O_2 and N_2, as well as atmospheric air (ATM), were

used as vehicles to mix the sample volume and maintain pO_2 during exposures. Oxygen tension was monitored before and after microwave and sham exposures using a Clark oxygen electrode: O_2, 760 mm Hg; N_2, 0 mm Hg; ATM, 155 mm Hg. No change in pH was observed during treatments.
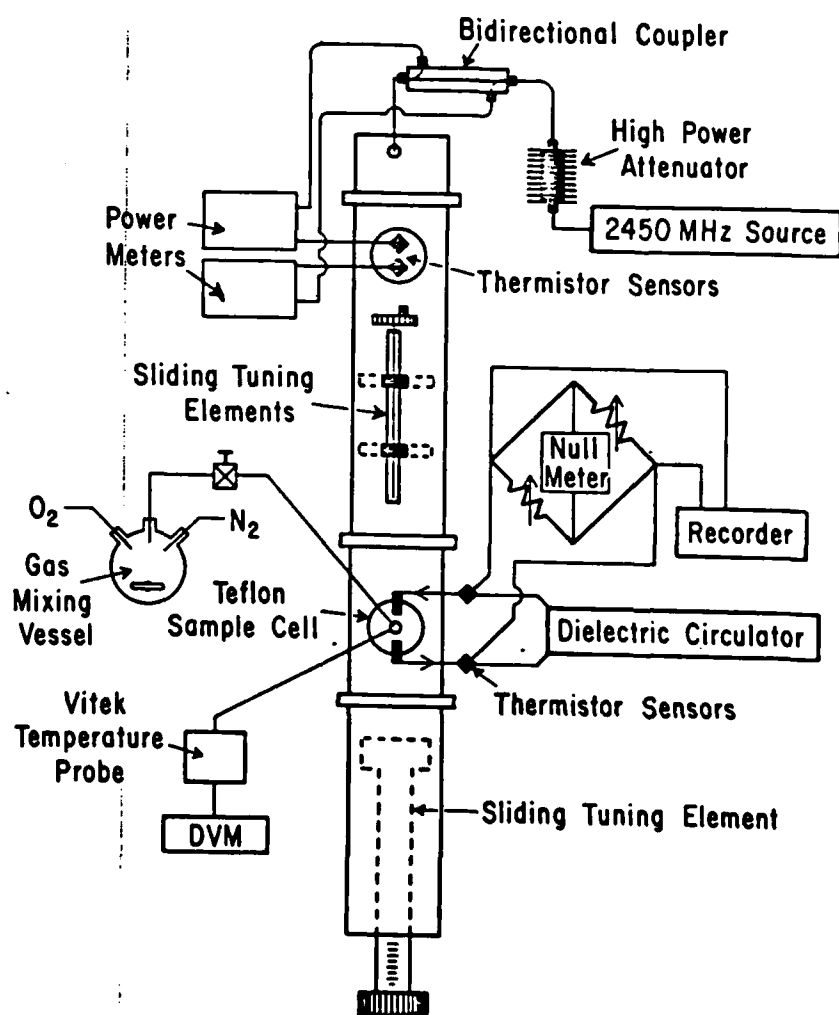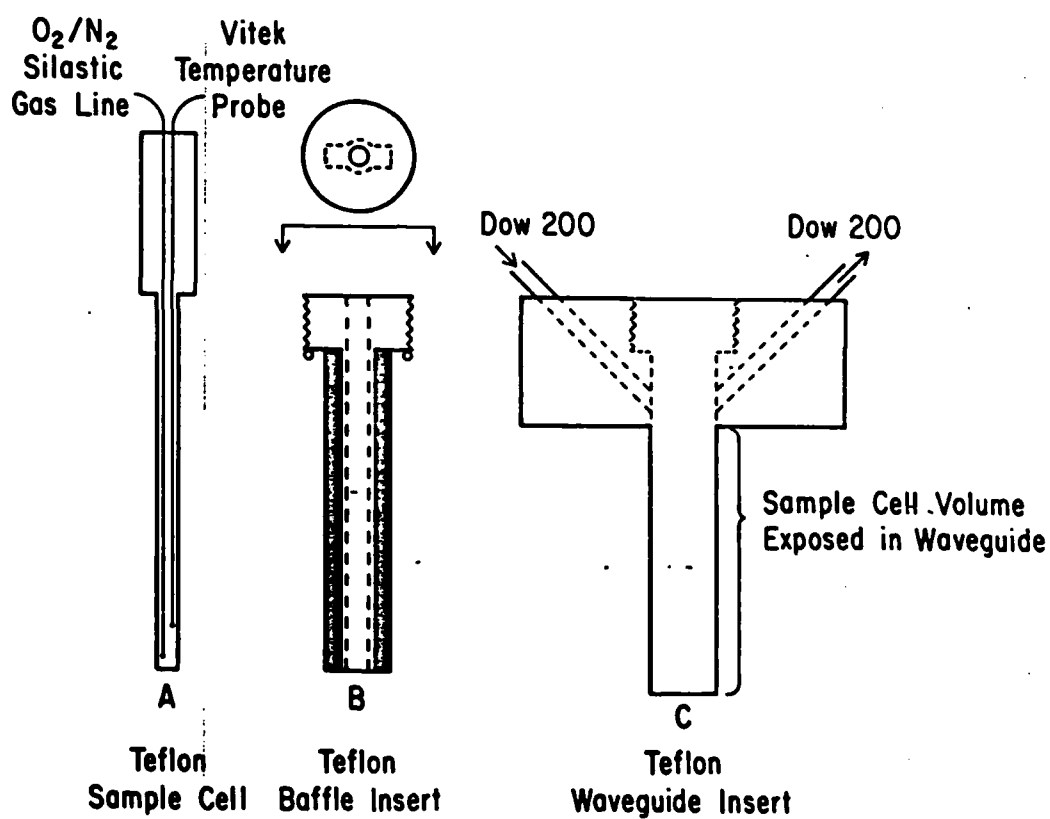
FIGURE 1a

FIGURE 1b

A
Teflon
Sample Cell

B
Teflon
Baffle Insert
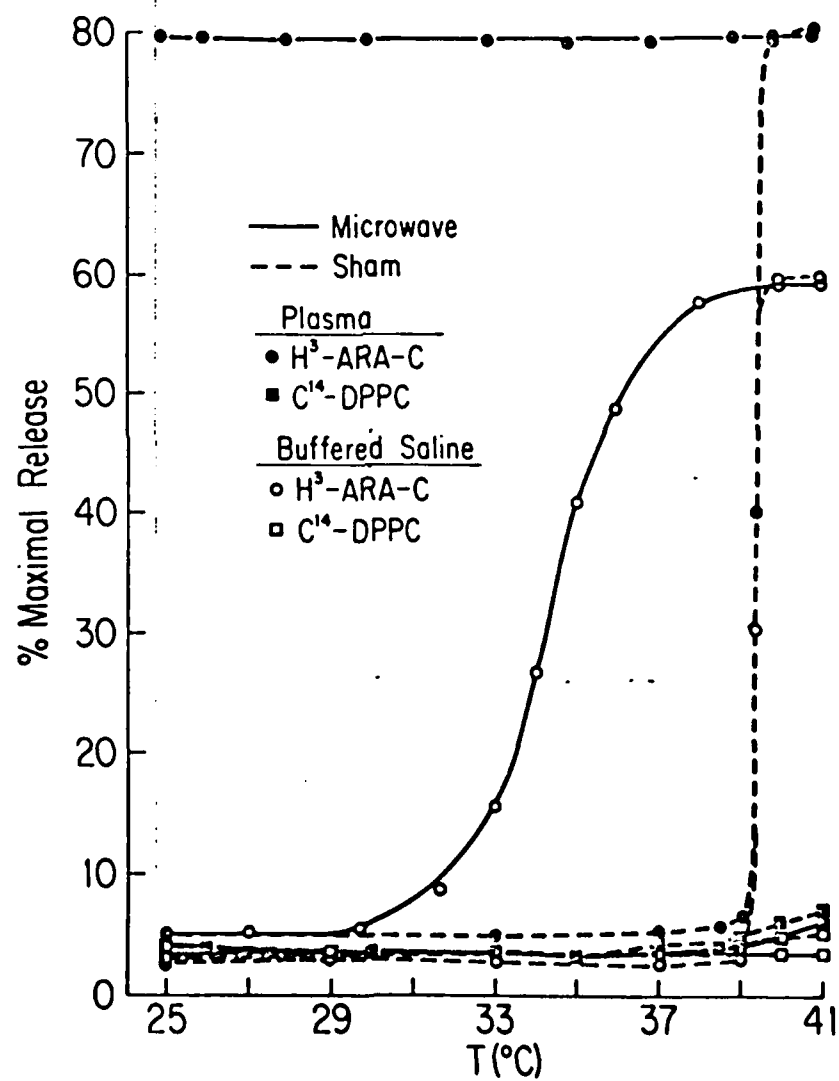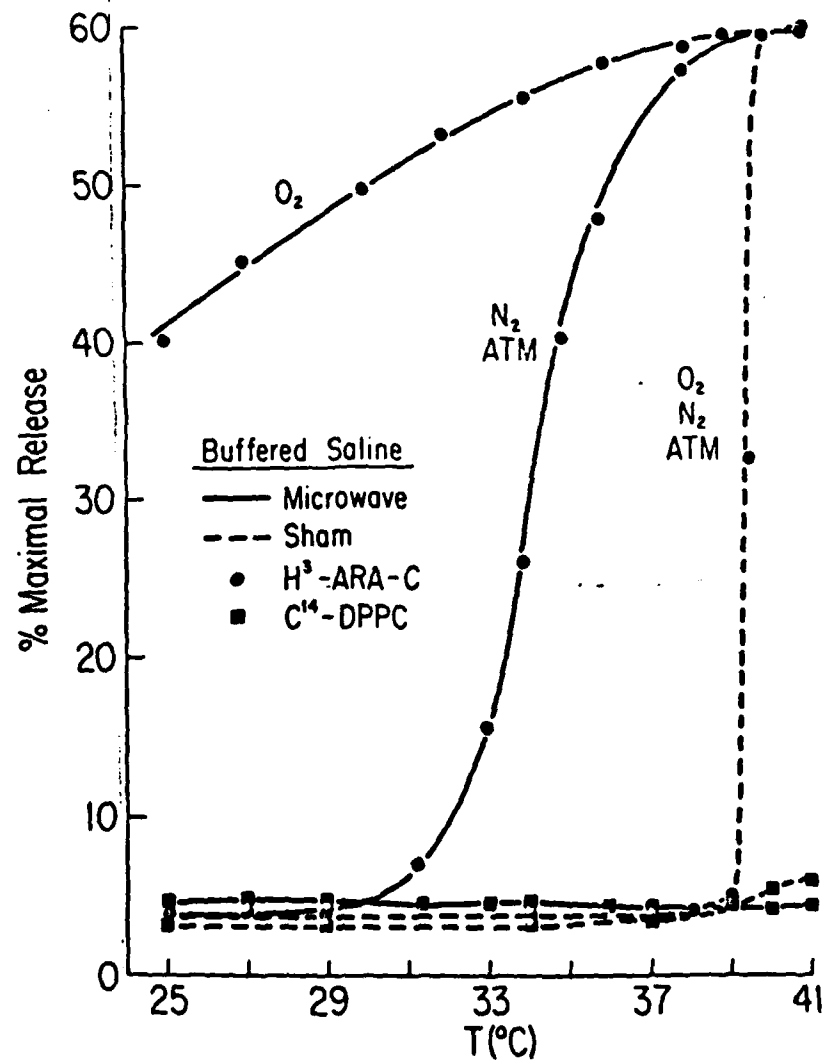
C
Teflon
Waveguide Insert

FIGURE 2a

FIGURE 2b

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