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A study to determine the effect of pulsed electromagnetic energy upon braincalcium behavior was undertaken. An innovative approach for loading the cerebral tissues with radiocalcium was indtroduced. Intraventricular injections through the skull placed Ca-45 solution directly into the right lateral ventricle. Two hours later companion frontal lobe samples were placed in separate glass beakers containing physiologic solution for a 20 min exposure to pulsed (26 Hz prf) electromagnetic energy (10 mw/cm squared at 2.45 GHz). An efflux value was calculated for each sample. A second experimental procedure involved whole-body irradiation of the animals two hours following the intraventricular injections. Animals were then irradiated with pulsed electromagnetic energy at a power density of 10 mW/cm squared, a pulse repetition frequency of 16 Hz, and carrier frequency of 2.45 GHz. Following exposure, frontal lobe and parieto-occipital tissue samples were taken and analysed for radioactivity. Statistical treatment of the first sets of experiments failed to reveal any perturbation in calcium efflux behavior. Data from the second set

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INVESTIGATION OF RADIO FREQUENCY/MICROWAVE EFFECTS UPON THE CENTRAL NERVOUS SYSTEM

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ABSTRACT

A study to determine the effect of pulsed electromagnetic energy upon brain calcium behavior was undertaken. An innovative approach for loading the cerebral tissues with radiocalcium was introduced in order to reduce the amount of tissue handling: intraventricular injections through the skull placed 40 µl of $^{45}Ca^{2+}$ solution directly into the right lateral ventricle. Two hours later, one of two experimental procedures was employed. In the first, the skull was opened and companion frontal lobe samples were taken from each animal and placed in separate glass beakers containing uncontaminated physiologic solution (efflux medium) for a 20 min exposure to pulsed (16 Hz pulse repetition frequency) electromagnetic energy consisting of one of three power density-carrier frequency schemes: 1 mW/cm^2 at 1 GHz, 10 mW/cm^2 at 1 GHz, and 10 mW/cm^2 at 2.45 GHz. Measurements of radioactivity in the efflux medium and in the tissue sample were used to calculate an efflux value (ratio) for each sample. Control samples were placed in the radiation chambre with the power off to accomplish sham-irradiation conditions. A second experimental procedure involved whole-body irradiation of the animals two hours following the intraventricular injections. The irradiated rat was restrained in a plexiglass holder and oriented such that the longitudinal axis of the animal was collinear with the k-vector and normal to the E-vector. The animal was then irradiated with pulsed electromagnetic energy at a power density of 10 mW/cm^2, a pulse repetition frequency of 16 Hz, and carrier frequency of 2.45 GHz. Following exposure or sham-exposure, frontal lobe and parieto-occipital tissue samples were taken and analysed for radioactivity.

Statistical treatment of the first sets of experiments have failed to reveal any perturbation in calcium efflux behavior. Data from the second set of experiments, employing whole-body irradiation, is still undergoing statistical analysis at this writing.

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INVESTIGATION OF RADIO FREQUENCY/MICROWAVE EFFECTS UPON THE CENTRAL NERVOUS SYSTEM

I INTRODUCTION

The increased use of radiofrequency (RF) and microwave power during the past two decades has spurred interest in ascertaining the biohazzard potential of these electromagnetic (EM) radiations. Research into the biological effects of EM radiations has expanded enormously in an effort to identify EM-induced biological deficits and describe the underlying mechanisms.

The central nervous system (CNS) has been a very prominent area of RF/microwave studies, and numerous reports exist proclaiming both the presence and the absence of low-level RF/microwave - induced alterations in the anatomical, neurochemical, and physiological aspects of the CNS (1,2,3,4,5,6), and some behavioral modifications at higher power levels has been noted (1). However, much research remains to be completed before these findings can be completely assessed.

An important consideration of the research outlined above is the effect of weak fields upon cerebral cation behavior. In particular, the profound role of the Ca^{2+} ion in the regulation of membrane resting potential and in synaptic events dictates that the influence of EM energy upon its neuronal distribution and kinetics be fully assessed.

Bawin and colleagues (4,7) have reported a range of frequencies (6-20 Hz) whose sinusoidal modulation (double sideband-large carrier, or DSB-LC, at 80-90% modulation depth) of a 147 MHz carrier could produce significant increases in $^{45}Ca^{2+}$ efflux from neonatal chick brain exposed <u>in vitro</u> at low power densities (PD) of around 0.8 mW/cm^2. The maximal response occurred at 16 Hz, where $^{45}Ca^{2+}$ efflux was found to be 18.5% above control. Modulating frequencies outside this

range were found to be ineffective in producing efflux changes. Furthermore, the direct application of the unmodulated carrier signal itself also failed to induce efflux perturbations. A subsequent report by Bawin and Adey (1976) considered the actions of only the slow modulation frequencies on $^{45}Ca^{2+}$ efflux. They showed that direct application of 6-16 Hz sinusoidal fields produced a decrease in $^{45}Ca^{2+}$ efflux in chick and brain tissue. Other studies by the Bawin group have shown that a similar increase release of $^{45}Ca^{2+}$ from chick cerebral tissue can be induced by a 450 MHz carrier signal amplitude-modulated at 16 Hz (8). The essential results of these investigations have been confirmed elsewhere (9).

Shelton and Mettitt (10,11) undertook parallel studies employing pulsed-mircrowave irradiation of rat cerebral tissues in vitro in the far-field region. The exposures performed consisted of several PD-PRF combinations (0.5, 1.0, 2.0 and 15 mW/cm^2 at 16 Hz; 1.0 and 2.0 mW/cm^2 at 32 Hz) with a 1 GHz sinusoidal carrier. No significant changes in $^{45}Ca^{2+}$ efflux could be elicited.

Thus far, experiments dealing with the response of brain calcium to modulated sinusoidal waveforms have been performed entirely under in vitro conditions, and the results must be interpreted with that in mind. A more attractive approach would be to perform as much of the experimentation as possible under in vivo conditions.

The present report, which is the Final Report on Minigrant AFOSR 79-0110, presents the findings of $^{45}Ca^{2+}$ efflux studies which incorporated in vivo techniques into the experimental procedure.

II EXPERIMENTAL

Introduction

In the study of brain calcium response to electromagnetic (EM) energy, the *in vitro* methods used exclusively to date have required that the brain be removed from the cranial vault and placed in a $^{45}Ca^{2+}$ -treated physiologic medium for 20 minutes or longer to accomplish radioisotope loading. Aside from the inherent shortcomings of *in vitro* methods in general, Bawin and Adey (7) have pointed out the sensitivity of calcium efflux in cerebral tissues to tissue handling. They noted that loading of the tissues, under *in vitro* conditions, takes place predominantly in the superficial regions of the tissues and that substantial variations in the efflux occur in response to changes in tissue handling procedures such as cutting and rinsing. This effect was confirmed by Shelton and Merritt in similar experiments.

The experimentation described in this report was designed to introduce more *in vivo* methodology into calcium efflux experimentation. Primarily, earlier experimentation by Shelton and Merritt (10,11) was repeated with the exception that *in vivo* tissue loading of $^{45}Ca^{2+}$ was introduced, and an additional carrier frequency of 2.45 GHz was added to the exposure parameters. The principal *in vivo* technique introduced in these experiments was the injection of the $^{45}Ca^{2+}$ through the skull directly into the right lateral ventricle of each animal and allowing time for sufficient incorporation of the radioisotope into the brain calcium population. Though the radiocalcium *loading* was *in vivo*, the *irradiation* was still performed under *in vitro* conditions. Thus, beyond the principal objectives, it was decided to pursue, additionally, *whole-body irradiation* of the of the animals in order to reduce further the amount of tissue handling.

Preliminary Experimentation

A preliminary set of experiments was performed to determine the efficacy of direct intraventricular injections. Initially, injections of trypan blue were made (with the animals under light ether anesthesia) and the animals sacrificed at various times up to 24 hours. These studies confirmed by visual inspection that injections targeted for the ventricles could be easily and reliably accomplished from outside the skull. No evidence of behavior modification was observed among the rats up to the time of sacrifice.

The second phase of the preliminary experiments was designed to establish a reasonable post-injection sacrifice time for the animals. Intraventricular injections of $^{45}Ca^{2+}$ -treated physiologic medium (see below) were followed by sacrifices at 2,4,8, and 48 hours after injection and frontal lobe tissue samples were assayed for radioactivity. This provided a sketch of the frontal-lobe $^{45}Ca^{2+}$ -uptake kinetics. Uptake of $^{45}Ca^{2+}$ was found to be approximately 25% complete by 2 hours following the injection. This figure was considered to be sufficient for counting purposes and, hence, a standard two hour post-injection sacrifice time was settled upon.

Intraventricular Injections

Male Sprague Dawley rats, 150-250 g, were used exclusively and allowed Purina Chow and water ad libitum. The animals were lightly anesthetized with ether in a closed container. They were then removed and a longitudinal incision was made along the midline of the scalp. The scalp was retracted and a vertical puncture (approximately 1 mm diameter) was made through the skull and into the cortex. A microsyringe with a 23 gauge needle (approximately 2.5 mm long) was immediately positioned over the puncture wound to permit the injection of 40 µl of the radioisotope solution through the puncture wound into the right lateral

ventricle. The injection was made gradually over a 2-3 second interval and pressure then briefly applied over the injection site prior to closing by suture. Characteristically, the injection procedure required 5-6 minutes following the anesthesia, with the animals recovering during the suturing.

Acquisition of Tissue Samples

For the in vitro exposures, the animals were sacrificed via cervical dislocation two hours following the intraventricular injections. The cranial vault was quickly opened and the entire brain removed and washed briefly with cold physiologic solution. Two frontal lobe samples from each animal were excised by a single coronal slice made approximately 5 mm posterior to the frontal poles (using a #10 surgical blade) followed by a midsaggital cut (when needed) to complete the separation of the samples. Each sample was weighed and placed in a 50 ml glass beaker containing 2 ml of the physiologic solution in preparation for irradiation of sham irradiation. Four such pairs of frontal lobe tissue samples were processed together in each exposure or control experiment.

Samples for the in vivo irradiation were taken immediately following irradidation or sham irradiation. The cranial vault was opened and the entire brain removed. Frontal lobe samples were taken in the manner described for the in vitro exposures. Additionally, a wide strip of cortial tissue from the middle half of the cerebrum was also taken.

Exposure of Samples

Irradiation was performed in the 10 ft x 10 ft test center of an Emerson and Cumming 10 ft x 10 ft x 24 ft tapered Eccosorb anechoic chambre located in the Radiation Sciences Division of the USAF School of Aerospace Medicine at Brooks AFB, Texas. The output of MCL Model 15022 microwave generator was trans-

mitted to a standard gain horn by flexible waveguide. Horn transmitters operating in the TEM mode were used for irradiation, with the animals located sufficiently distant to ensure far field conditions. Incident power densities at the irradiation pane were measured with a Narda Electromagnetic Radiation Monitor (Model 8316 b) and Isotropic Probe (Model 8231). The chambre was modified to permit environmental control which ensured consistent chambre temperature and humidity throughout the experiments.

For the in vitro exposures, standard gain horns were mounted above the samples so that normal incidence of the EM wave upon the plane of the samples was achieved. For each in vitro exposure or sham exposure, the eight sample-containing beakers were arranged in two parallel rows of four beakers each. The beakers were restrained in a styrofoam holder which was afloat in a plexiglas waterbath. The lower part of the holder (and, therefore, the bottoms of the beakers) was submerged and was designed to permit circulation of the temperature-controlled water around the beakers. The circulation was promoted by the transverse flow of the water from the inputport of the water bath to the output port and by a gentle lateral oscillation imparted to the styrofoam holder by a long plexiglas rod connected to the movable section of another water-bath maintained outside the irradiation area. The lateral oscillation frequency was approximately one cycle every two seconds, and the lateral displacements of the holder were restricted to ensure that the samples were at all times within the region of constant power density. The water was routed via tygon tubing to and from a Neslab heating unit stationed just outside the anechoic chambre. The water was kept at 37°C in the large reservoir of the Neslab unit, and the temperature continuously monitored by visual insepection during the exposure period. The temperature of the water in the water bath within the chambre was monitored by a

Vitek electrothermia monitor (Model 102). The sensor leads were fed into the water bath from outside the irradiation area and attached to the side of the water bath by tape such that they were immersed in the adjacent water. The output of the Viteck electrothermia monitor was fet outside the chambre to an HP 9830 A which was programmed to record the temperature measurements at 30 sec intervals.

In the in vitro irradiation experiments, a 16 Hz PRF was selected on the basis of its "analogy" to the 16 Hz sinusoidal modulating frequency found by Bawin and Adey (4) to be optimal in producing changes in cerebral calcium efflux in DSB-LC experiments. Power densities of 1 mW/cm^2 and 10 mW/cm^2 at 1.0 GHz were also related to the work of Bawin and Adey (4) which indicated that the former PD might be biologically effective. Two additional sets of experiments utilizing the 16 Hz PRF and the same two PD figures at a carrier frequency of 2.45 GHz were included in the experimental plan in order to provide a shorter wavelength of biological importance. In these latter experiments only the 10 mW/cm^2 irradiations were performed, since it was then decided to proceed to the whole-body experiments and return later to complete the final in vitro exposure experiment. The whole-body experiments subsequently occupied the remainder of the experimentation and the final in vitro experiment had to be delayed until the next opportunity arose in the lab. Whole-body experiments are still in progress at USAFSAM due to continuing interest by personnel in the Radiation Sciences labs. Upon their completion, the final in vitro experiments will be pursued.

The selection of parameters for the whole body irradiations were made on the same basis as described for the in vitro experiments. A summary of all the experiments is presented in Table 1.

In vivo, or whole-body, exposures were begun at the two hours post-injection times. The rats were placed in a cylindrical, plexiglas restrainer which rested horizontally on a styroforam platform, with the longitudinal axis of the animal aligned with the incident k-vector and normal to the E-vector in the far-field region. During the exposure of sham-exposure, the rectal temperatures were monitored with a Vitek electrothermia monitor. The output was fed into an HP 9830A outside the chambre which recorded the temperatures at 30 sec intervals.

Experiment	Exposure	PRF (Hz)	PD (mW/cm^2)	Carrier Freq. (GHz)
I	in vitro	16	1.0	1.0
II	in vitro	16	10.0	1.0
III	in vitro	16	10.0	2.45
IV	in vivo	16	10.0	2.45

Table 1. Summary of Experiments

Injection Solution

The solution used for intraventricular $^{45}Ca^{2+}$ loading was modeled after Cooke and Robinson (12): 124 mM NaCl, 26 mM $NaHCO_3$, 5mM KCl, 12 mM KH_2PO_4, 1.3 mM $MgSO_4$, 0.75 mM $CaCl_2$, 10 mM glucose and 2 μCi of $^{45}Ca^{2+}$. For the in vitro exposures, the uncontaminated version of the medium was used for efflux analysis.

Radioassay of Samples

Upon completion of the 20 min exposure period for the in vitro experiments, a 0.5 ml aliquot of the physiologic medium was pipetted into a counting vial and the tissue sample washed briefly with fresh physiologic solution and also placed in a counting vial. The tissue sample was digested overnight in 2 ml of Soluene

in an oven maintained at 37°C. A 1 ml aliquot of the resultant solution was transferred to a new counting vial and 9 ml of Dimulume was added. The 0.5 ml aliquots of the physiologic medium were also supplemented with 9 ml volumes of Dimulume at this time. The vials were then placed in a Beckman LS 250 liquid scintillation counter and counted for 10 min periods to determine radioactive content.

For the in vivo exposure experiments, the samples were treated overnight in Soluene and then processed in the same fashion as the in vitro samples.

Calculations and Statistics

For the in vitro exposure experiments, the calcium efflux was calculated as the ratio

$$\text{efflux} = \frac{\text{CPM (medium)}}{\text{CPM (tissue)} + \text{CPM (medium)}}$$

where CPM (medium) is the total counts per minute in the efflux medium as determined from radioassay of the aliquots and CPM (tissue) is the total CPM measured in the tissue at the end of an experiment. The denominator, then, represents the total CPM in the tissue at the outset of an exposure. All counting was corrected for radioisotope half-life and efficiency of the counting system. The efflux values of the irradiated tissues were compared to the companion sham-irradiated tissue using the student's t-test. Nonsignificance of difference was assumed if $P>0.05$.

Data from the in vivo exposures is still being processed. At this time, the data is being transformed into a simple number representing CPM per g of brain tissue.

III RESULTS

The results of the *in vitro* exposure experiments are summarized in Table 2, and are presented graphically in Figure 1.

Experiment Number	Efflux Value (m ± S.D.) Radiated (n)	Control (n)	t-test
I	5.70± 1.57 (46)	6.15± 1.25 (53)	P>0.05
II	4.98± 1.44 (22)	5.04± 1.06 (22)	P>0.05
III	6.42± 1.63 (24)	5.70± 1.33 (22)	P>0.05

Table 2. Summary of *in vitro* exposure experiments. Efflux values are expressed as mean percentages (m) and standard deviations (S.D.), and the number of samples (n) used in calculating each value is given in parentheses.

At the time of this writing, whole-body irradiation (Experiment IV) using a power density of 10 mW/cm^2 and a 2.45 GHz carrier frequency has been completed, but data processing and analysis is still being performed.

For the *in vitro* irradiation results presented here, the differences between radiated and sham radiated means was considered significant if the t-test yielded $P>0.05$. All experiments completed so far indicate nonsignificant differences in the means since $P>0.05$.

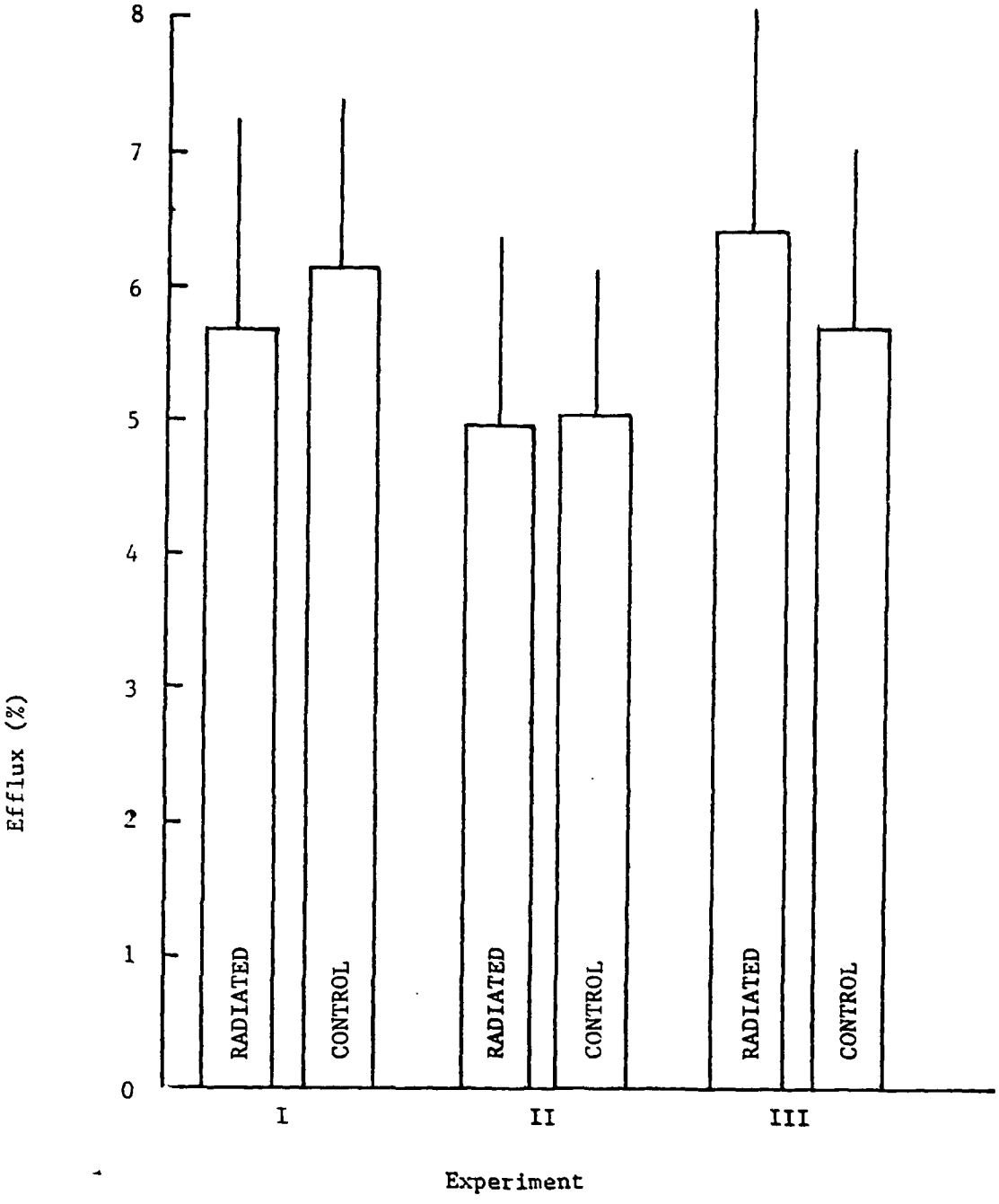

Figure 1. Calcium efflux value comparisons between radiated and control samples for the in vivo irradiation experiments. Vertical bars indicate one standard deviation.

IV DISCUSSION

The introduction of intraventricular $^{45}Ca^{2+}$ injections to permit *in vivo* exchange of the isotope with the indigenous brain calcium represents a significant improvement in calcium efflux studies. Equilibration thus could proceed under more homeostatic conditions and eliminated were the tissue handling procedures prior to $^{45}Ca^{2+}$ loading of the tissue as well as the in vitro loading technique itself. The intraventricular injection technique was found to be a reliable and reasonably easy method of delivering radiocalcium directly into the brain at a location proximal to the frontal lobes from which the samples were to be taken. The 2 hour post-injection interval required for accumulation of the isotope in the frontal lobes added 1 2/3 hour to the time utilized in the *in vitro* loading procedure. This factor along with the innovative intraventricular injection made the overall procedure more complicated and time consuming, and dictated a rigorous schedule for each day's experiments. However, the enhancement in experimental design more than offset the sacrifice in the rate at which data could be collected.

Whole-body irradiation following intraventricular delivery of radiocalcium perimtted brain calcium kinetics to proceed with the homeostatic mechanisms remaining operational up to the time of sacrifice of the animals. However, the rate of data collection was reduced substantially as a consequence of the constraint that only one animal at a time could occupy the irradiation chambre for either exposure or sham exposure. Furthermore, a new expression for quantifying the efflux ratio had to be employed since there now existed no efflux solution.

The *in vitro* irradiations performed in this experimentations failed to produce statistically significant changes in cerebral calcium efflux. These findings confirmed the conclusions drawn from previous experiments (10,11) involving

the same exposure parameters (1.0 and 10.0 mW/cm^2 at 16 Hz PRF and 1.0 GHz carrier frequency), and extended consideration to higher carrier frequencies (2.45 GHz) where the same findings were seen.

Although no significant differences in efflux values were found betweem exposed and control rats in this experimentation or between exposed and control rats in the previous experiments (10,11), it should be noted that a marked difference in the efflux values derived from these two experimental methodologies does occur. Shelton and Merritt (10,11) previously reported efflux values of 36.8 % (exposed) and 39.5 % (control) for an experiment corresponding to experiment I of this report. This is seen to be in dramatic contrast to the figures of 5.7 % and 6.15 %, respectively, derived from the present experimentation. These differences apparently develop from such factors as cerebral vascularization and CSF flow (as well as slightly modified tissue cutting procedures) as compared to the immersion-type of treatment involved in the <u>in vitro</u> method radioisotope loading. The $^{45}Ca^{2+}$-rich superficial regions of tissues loaded by the latter method thus appear capable of releasing of greater amounts of the radioisotope into the efflux medium during the 20 min effluz period. This tendency of <u>in vivo</u>-loaded tissue to retain $^{45}Ca^{2+}$ may also reflect a more genuine incorporation of the radioisotope into normal calcium roles in the brain.

Experimental animal and carrier frequency differences aside, it is perhaps appropriate here to point out a few of the other fundamental differences between the exposure techniques employed in the present work and those used by other researchers to date (4,5,6,7,8). The use of pulsed microwave energy with a 0.32 duty factor means that the samples were actually being irradiated only about a third of the time during the 20-minute exposure period. This contrasts with the continuous exposure in the double sideband-large carrier (DSB-LC) tech-

nique used by others. Of course, the response of tissue to the abrupt on-off fields of pulsed radiofrequency (RF) energy, as opposed to the steady-state sinusoids, is another point of consideration. Finally, the use of DSB-LC implies a signal magnitude spectrum consisting of a carrier component along with a sideband component on either side at a separation distance equal to the modulating frequency. For 80-90% modulation, simple calculations place some 24-29% of the transmitted power in the two sidebands, which are suggested by others to be the biologically significant components. Pulsed RF radiation on the other hand, is characterized by a magnitude spectrum consisting of a carrier term and a number of symmetric sidebands spaced about the carrier at integral multiplies of the PRF and following a $\frac{\sin x}{x}$ envelope.

The process by which a membrane might demodulate single-tone DSB-LC signals is unresolved, but suggestions for an exquisitely sensitive mechanism have been offered recently (see Adey 1977 for discussion). This mechanism represents a highly selective membrane response to electromagnetic energy according to the constraints imposed by the "power window" phenomena. Thus, given the contrasting properties of pulsed RF fields, the question arises as to whether such a signal (pulsed RF) possesses any of the requisite characteristics for triggering a demodulation mechanism. Further , if the pulsed RF signals were in fact somehow demodulated, it would still remain to show that the tissue interprets the recovered pulse-train the same as it would a sinusoid recovered from a DSB-LC signal.

The results presented in this report reinforce the conclusions of earlier work (10,11) that if a pulsed RF demodulation mechanism exists in rat cerebral tissue, it is either unresponsive or else insufficiently responsive to the

pulsed RF parameters defined in Table 1.

The need for additional research in this area is apparent. Of immediate interest would be an experiment designed to place, in the first sidebands of the pulse spectrum, an amount of energy equivalent to that used in DSB-LC experimentation, since it is this energy that is suggested to be biologically potent. Additional combinations of carrier frequency, PRF, power density, and other types of modulation need to be incorporated into this experimentation.

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