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EFFECTS ON ELECTROMAGNETIC FIELDS ON CALCIUM EFFLUX
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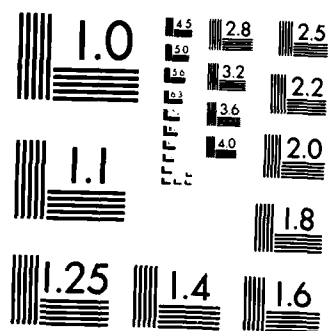
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FINAL TECHNICAL REPORT

EFFECTS ON ELECTROMAGNETIC FIELDS ON CALCIUM EFFLEUX FROM
MONOLAYER CULTURES OF BRAIN CELLS ISOLATED FROM EMBRYONIC CHICK CEREBRAL HEMISPHERES

Contract N00014-80-C-0363

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Submitted by:

Lester M. Partlow, Ph.D., and
L. J. Stensaas, Ph.D.

Department of Pharmacology

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STIMULATION OF 2-DEOXYGLUCOSE UPTAKE BY SELECTED BRAIN REGIONS

IN RATS IRRADIATED WITH 2450 MHz MICROWAVES

L. M. Partlow, L. G. Bush, A. Riazi, J. D'Andrea, C. W. Turner, and O. P. Gandhi, Departments of Pharmacology (LMP and LGB), Electrical Engineering (AR, JD, and OPG), and Psychology (CWT), University of Utah, Salt Lake City, Utah 84132

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ABSTRACT

Rats were injected with labelled 2-deoxyglucose (70 nCi/gm) through the femoral artery and immediately placed in a plastic enclosure which served to partially align the long axis of the animal's body with the either the E- or K-vectors associated with the microwave field. Rats were then irradiated at 2450 MHz for 40 min, sacrificed by an i.p. injection of a lethal dose of phenobarbital, and perfused intracardially with isotonic fixative to wash unincorporated 2-deoxyglucose out of the vasculature. Brains were rapidly removed and cut into 10 regions. Each region was weighed and then dissolved in Protisol prior to scintillation counting. Levels of uptake in DPM/mg wet weight were normalized to adjust for small differences in injected 2-deoxyglucose.

Microwave irradiation of alert rats at 2450 MHz in both the K||L and E||L orientations significantly increased 2-deoxyglucose uptake in the rostral and dorsal portions of the brain. Irradiation of alert rats in the K||L orientation caused a smaller increase in 2-deoxyglucose uptake than irradiation in the E||L orientation (6.6% and 14.4%, respectively). No microwave-induced changes in 2-deoxyglucose uptake were observed either in the cerebellum or in any basal portion of the CNS caudal to the optic chiasma.

Incorporation of 2-deoxyglucose by anesthetized rats was reduced by 17% relative to that in alert rats. Microwave irradiation of anesthetized rats aligned with either the E- or K-vector was without effect on 2-deoxyglucose uptake into any region of the rat brain.

INTRODUCTION

Numerous reports have indicated that microwave irradiation might pose a significant health hazard to man. For example, Soviet and Polish investigators have identified a syndrome stemming from acute exposure to low levels of microwave irradiation [$\leq 10 \text{ mW/cm}^2$], which is characterized by "headaches, nausea, vertigo, and sleep disturbances . . . (and by) . . . hypertonia, changes in cardiac rhythm, skin rash . . . (and) a decrease in the amplitude of alpha waves" upon EEG examination at low power densities [1]. They have concluded that exposure to microwaves at low power densities can lead to serious disturbances both in the central and in the peripheral nervous system [1]. Similar allegations have recently been made in a controversial book published in this country [2]. In contrast, most Western investigators question the claims made by their Soviet and Polish colleagues [3,4,5] and feel that the available evidence is insufficient to allow any firm conclusions to be reached at this time.

Studies on experimental animals provide some support for the idea that microwave irradiation can significantly affect the central nervous and cerebrovascular systems. Microwave-induced hyperthermia has been shown to cause (a) convulsions and death [6,7], (b) focal areas of brain necrosis [6,8], (c) increased permeability of the blood-brain barrier [9, 10, 11, 12], (d) vasodilatation both in the central nervous system and the periphery [6,8], and (e) an increased rate of respiration [13]. All of these effects can be ascribed directly to cerebral hyperthermia. For example, focal areas of necrosis in the brain could result from a thermal enhancement of platelet aggregation resulting in thrombosis, anoxic ischemia, and localized tissue destruction. Similarly,

brain convulsions could result from a generalized increase in neuronal activity and/or irritability brought about by the increased temperature [14]. Furthermore, Merritt has shown that the increased permeability of the blood-brain barrier might be due solely to the microwave-induced hyperthermia [9]. Finally, both peripheral vasodilatation and an increased respiratory rate could result from warming a small population of neurons in the anterior hypothalamus which are normally involved in regulating body temperature [15]. Thus, all of these effects could be explained by heating of the brain.

In contrast, several reported microwave effects that bear on the nervous system might conceivably operate by athermal mechanisms (i.e., those occurring without a detectable rise in temperature). Thus, Lords [16, 17] has reported that low levels of microwave energy can alter the function of a cholinergic synapse in the rat and the turtle hearts. Since millions of similar synapses exist within the nervous system, this study indicates that microwaves might have selective effects on the neurotransmitter mechanisms in the brain. In addition, McRee [18] has reported that athermal microwave effects caused a decrease in the ability of frog sciatic nerves maintained in vitro to sustain a high rate of firing over a prolonged period. This loss of vitality in microwave-treated nerves could have a profound effect on brain function. The possibility that low levels of microwave irradiation might have physiologically significant athermal effects on nervous tissues raises many currently unanswerable questions about the appropriateness of existing safety standards.

Numerical solutions have been obtained for isolated heads and for heads attached to bodies which provide two significant insights into microwave absorption by the brain. First, studies by Guy [19] and others [20, 21] on isolated spherical models of the head have shown that significant focusing effects at certain wavelengths result in a nonuniform absorption of microwave energy. Hot spots were predicted and later confirmed in models which absorbed 8 to 10 times as much microwave energy as the rest of the head. A spotty distribution of absorbed energy might produce highly localized areas of extreme hyperthermia. Such a nonuniform energy distribution might well explain Albert's observation that alterations in the blood-brain barrier were scattered throughout the brain [22]. Second, Gandhi [23] has shown that at certain frequencies the head absorbs 3.6 to 4 times as much microwave energy as the rest of the body by both numerical solutions for head-body models and by experimental measurements on models. Thus, Gandhi's results suggest that the brain might be peculiarly liable to injury by low levels of microwave irradiation at or near head-resonant frequencies.

These results indicate that microwave irradiation might have both thermal and athermal effects on brain function and also reveal the possibility that the brain might be peculiarly vulnerable to such an insult. The present study is designed to determine whether irradiation of rats at 2450 MHz, a frequency near the calculated head-resonance frequency for this species, will alter the local extent of neuronal activity as determined by Sokoloff's 2-deoxyglucose technique [1978]. This technique quantifies local patterns of glucose uptake by use of autoradiography to measure the intracellular accumulation of labeled 2-deoxyglucose-6-phosphate in different areas of the brain 40 min after i.v. administration of labeled 2-deoxyglucose.

MATERIALS AND METHODS

Animals and Animal Handling

Long-Evans rats (200 gm) will be employed in all studies. Animals will be behaviorally habituated to restraint in the radiation chamber for 60 minutes once each day for the week prior to irradiation. On the day of irradiation, each animal lightly anesthetized using penthrane and injected with an isotonic saline solution containing 70 nCi/gm 2-deoxy-D-[U-¹⁴C]glucose (Amersham CFB.181; 300-350 mCi/mmol) via the femoral artery. (Immediately prior to use, the 2-deoxyglucose was freeze dried and resuspended in sterile isotonic saline in order to remove the 3% ethanol.) After several minutes, the animal recovered from the gaseous anesthetic and was placed into the irradiation chamber and restrained, as before, in a relatively microwave-transparent plastic animal holder [24]. After the 40-minute period of microwave exposure or sham irradiation, the animals were sacrificed by i.p. injection of pentobarbital (35 mg/kg). Since pentobarbital has been shown to profoundly and uniformly depress the level of 2-deoxyglucose uptake throughout the brain [25], injection of this drug should also inhibit further uptake of 2-deoxyglucose by the brain following the radiation period. Animals were fixed by cardiac perfusion with cold fixative in order to partially fix the brain and to remove all 2-deoxyglucose remaining in the blood (ca 30% of initial plasma level) following the 40 min exposure period.

The brain and an attached rostral segment of spinal cord were rapidly removed. Each brain was placed in a pool of saline on a sheet of glass, cleaned of any adhering membranes, and cut into 10 portions as shown in Figure 1 using a razor blade. Each section was carefully blotted, weighed, and placed in a scintillation vial containing 1 ml of Protisol. After digestion, 20 ml of scintillation fluid (Aquasol II) was added and a week was allowed for chemoluminescence to disappear prior to scintillation counting to determine the amount of labelled 2-deoxyglucose present in each brain region in terms of DPM/mg wet weight.

Irradiation Conditions

Each animal was individually irradiated in a relatively microwave-transparent Plexiglas holder [24] placed in an anechoic chamber lined with pyramidal Eccosorb. A 13.5" x 19.5" pyramidal-horn antenna provided a measured beam width of $\pm 7.8^\circ$ at 2450 MHz in the E- and H- planes for field variables within ± 0.5 dB. A Toshiba 500 W magnetron was utilized with 2 meters from the horn's aperture to the cage. Since the animals were not in electrical contact with the vertical ground plane, the SAR has been shown by calorimetric measurements to be identical to that for plane wave irradiation.

The orientation of the animal with respect to the electric, magnetic, and propagational vectors is critical in determining the average peak SAR values in the exposed tissues. Rats were irradiated with (1) the long axis of the animal's body lying along the propagation vector (K||L) or (2) the long axis of the animal's body lying along the electric vector (E||L). On the basis of some recent work [23], a peak absorption in the 250 gm rat's head (amounting to about 25 percent of the whole body value) is anticipated at approximately 2300 MHz for both E||L and K||L orientations.

RESULTS

Incorporation of 2-deoxyglucose into the spinal cord. Six rats were injected with varying amounts of 2-deoxyglucose and samples of blood were drawn after periods ranging from 1 to 40 minutes. In addition, the rats were sacrificed after 40 min as described in Materials and Methods and a segment of spinal cord just caudal to the medulla (region 1, Fig. 1) was weighed and analyzed for labeled 2-deoxyglucose. Results from the series of timed plasma samples were plotted and regression analysis was used to obtain the best fit of the data to an equation having the form $Y = A + B \cdot \ln(X)$. The calculated Y-intercept (A) was taken as the best estimate of the plasma level of 2-deoxyglucose immediately after i.v. administration. The initial plasma level of 2-deoxyglucose was closely correlated ($r = 0.981$) with the amount of 2-deoxyglucose incorporated in the excised region of spinal cord.

Incorporation of 2-deoxyglucose into the spinal cord was also studied in order to determine whether it was affected by irradiation of either alert or anesthetized rats oriented in either the E||L or K||L orientations. The results shown in Table I demonstrate that uptake into this portion of spinal cord was unaffected by any treatment. A two (alert/anesthesia) X three (treatment condition: control, K||L irradiated, E||L irradiated) factorial analysis of variance was performed on the levels of uptake of 2-deoxyglucose into region 1. Neither the main effects for alert vs. anesthetized rats ($F(1,30)=1.127$; $P \geq 0.3$) nor for radiation condition ($F(2,30) < 1$) were significant. Thus, no evidence was obtained indicating that uptake of 2-deoxyglucose into this portion of the spinal cord was affected by any of the six conditions tested.

The standard errors shown in Table I demonstrate that the results exhibited considerable variability within the same treatment group. In the worst case, 2-deoxyglucose uptake varied within anesthetized rats irradiated in the E||L orientation from 17.2 to 59.9 DPM/mg with a mean and standard error of 29.5 ± 8.0 DPM/mg wet weight. Most of this variability is believed to be due to difficulties in reproducibly administering the precise i.v. dose of the radiolabel. Problems such as leakage of a portion of the dose out of the vasculature through the needle hole have been observed.

These results serve to illustrate the desirability of normalizing our data on 2-deoxyglucose uptake in order to minimize the variability due to extrinsic factors. Therefore, in all other experiments reported herein, values for uptake of 2-deoxyglucose have been normalized by adjusting the data set from each individual rat so that spinal cord uptake always equals 33 DPM/mg which is the overall mean for all treatment conditions in Table I. Normalization in this fashion is statistically justifiable since (1) spinal cord uptake is closely correlated with the initial plasma level of 2-deoxyglucose (Fig. 2) and (2) none of the treatments used in the present study had any effect on uptake in this region (Table I).

Incorporation into five dorsal and rostral portions of the CNS (regions 5, 7, 8, 9 and 10; Fig. 1) in the presence and absence of microwave irradiation. In alert animals, levels of uptake increased by a small amount with both K||L- and E||L-irradiation in all five regions in a similar manner (Fig. 3). A nested analysis of variance was carried out with three factors (radiation condition (3 levels), individual animals (6 levels), and separate portions of the CNS (5 levels)). The main effects for both radiation condition ($F(2,15)=10.33$; $P < 0.005$) and brain region ($F(4,60)=7.17$; $P < 0.005$) were significant. A strength of association measure was also calculated for both of these factors by dividing the sum of the squares associated with the factor by the total sum of the squares; this value indicates that 35% of all the variance is accounted for by

the treatment effect while an additional 12% is accounted for by the effect of brain region. A strength of association value of 0.35 is taken as indicating **■** a particularly strong effect. Finally, the interaction term was not significant ($F(8,60) < 1$).

These results can be interpreted as follows. First, uptake varied with portion of the brain, but this is not surprising since the neuroanatomy of the brain is heterogeneous and 2-deoxyglucose is incorporated in these regions to different extents depending on the cytoarchitecture and extent of neuronal activity. Second, uptake into all five regions of the alert rat brain varied with radiation condition. The main effects means for control, K||L, and E||L radiation conditions were 49.83, 53.13, and 56.98 DPM/mg, respectively. Thus, the average increases in 2-deoxyglucose uptake caused by K||L- and E||L-irradiation were 6.6% and 14.3%, respectively. The significance of these means from one another was assessed using the Newman-Keuls test which demonstrated that (a) control uptake differed significantly from that in both K||L- ($P=0.05$) and E||L-irradiated ($P<0.01$) animals. In addition, the Newman-Keuls test demonstrated that uptake of 2-deoxyglucose by the E||L-irradiated brains was significantly greater than that by the K||L-irradiated brains ($P=0.05$). Third, the lack of any significant interaction between radiation condition and brain region suggests that it is improbable that any single region is contributing a large portion of this main effect. Thus, it is most likely that all regions contribute rather uniformly to the observed differences. This conclusion is consonant with the data presented in Figure 3 on the means of the various treatment groups for each of the five brain regions. These results suggest that all brain regions behave similarly with regard to treatment condition and provide statistical justification for pooling these five regions.

In contrast, entirely different results were obtained when the effects of radiation on the same five regions in the brains anesthetized animals were investigated. The results shown in Figure 4 suggest that no significant differences exist as a result of radiation condition. A nested analysis of variance as described above was performed. The main effects for brain region was found to be significant ($F(4,60)=4.14$; $P=0.005$) but, as discussed above, this is not surprising. Neither the main effect for radiation condition ($F(2,15) < 1$) nor that for interaction between brain region and radiation condition ($F(8,60) < 1$) were significant. Thus, anesthesia appears to block the microwave-induced enhancement of 2-deoxyglucose uptake in brain regions 5, 7, 8, 9, and 10.

Incorporation of 2-deoxyglucose into brain regions 2, 3, 4, and 6 in the presence and absence of microwave irradiation. Examination of the data for alert (Fig. 5) and anesthetized rats (Fig. 6) suggests that microwave irradiation had no effect on 2-deoxyglucose uptake in either the cerebellum (region 3) or in the basal regions of the CNS caudal to the optic chiasm (regions 2, 4, and 6). Nested analysis of variance was separately performed on regions (2 + 3) and (4 + 6) both because this pairing seemed to correspond to a reasonable anatomical grouping of areas and because the patterns of response were very similar between these groups. For the alert animals (Fig. 5), the main effect for brain region was significant for both regions (2+3) and (4+6) ($F(1,15) < 18.5$ or 11.8 , respectively; $P < 0.005$). In contrast, neither the main effect for radiation condition nor the interaction effect between radiation treatment and brain region was significant for either pair of regions in these alert animals ($F(2,15) < 1$). For the anesthetized animals (Fig. 6), the results of a nested analysis of variance yielded similar results except that even the main effect for brain region was not significant for regions (4+6). In summary,

no effect ascribable to microwave irradiation was observed in CNS regions 2, 3, 4, and 6 from either alert or anesthetized rats irradiated in either orientation.

DISCUSSION

The results presented herein demonstrate that microwave irradiation of alert rats at 2450 MHz can cause an alteration in the uptake of 2-deoxyglucose in the frontal and dorsal portions of the CNS. This increase could result from (a) a direct heating of these CNS regions with a resultant increase in the brain's demand for glucose, (b) direct local heating with a resultant increase in neuronal activity which in turn increases demand for glucose, or (c) an indirect effect of glucose on some other portion of the body with an increased neuronal input to the affected brain regions which in turn increase their electrical activity and incorporate more 2-deoxyglucose. Additional data will be needed in order to determine which mechanism is active in producing a microwave-induced increase in uptake of 2-deoxyglucose.

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FIGURE LEGENDS

Figure 1. A diagrammatic illustration of the 10 regions into which the each rat brain was cut.

Figure 2. Relationship between the level of labeled 2-deoxyglucose found in the plasma immediately after i.v. administration and the amount of 2-deoxyglucose incorporated into the segment of spinal cord caudal to the medulla during the next 40 min. Different amounts of 2-deoxyglucose were injected into the femoral vein and the rats were sacrificed after 40 min as described in Materials and Methods. Linear regression analysis demonstrated that these two measures were closely correlated and that the best fit equation of the form $Y = a + b \cdot X$ was obtained if $a = -8.5 \pm 19.4$ and $b = 2.57 \pm 0.26$ ($n = 6$).

Figure 3. Uptake of 2-deoxyglucose into five brain regions in alert rats which were either sham irradiated or microwave irradiated with the long axis of the animal's body parallel to either the electric (E||L) or propagation vector (K||L). Values represent means $\pm$ the standard error ($n=6$); the brain regions numbered herein correspond to those shown in Figure 1. All brain regions respond similarly to irradiation and exhibit an average +6.6% increase in uptake as a consequence of K||L irradiation and a +14.3% increase as a consequence of E||L irradiation.

Figure 4. Uptake of 2-deoxyglucose into five brain regions in anesthetized rats which were either sham irradiated or microwave irradiated with the long axis of the animal's body parallel to either the electric (E||L) or propagation vector (K||L). Values represent means $\pm$ the standard error ($n=6$); the brain regions numbered herein correspond to those shown in Figure 1. All brain regions failed to respond to irradiation as judged by individual T-tests.

Figure 5. Uptake of 2-deoxyglucose into four brain regions in alert rats which were either sham irradiated or microwave irradiated with the long axis of the animal's body parallel to either the electric (E||L) or propagation vector (K||L). Values represent means $\pm$ the standard error ($n=6$); the brain regions numbered herein correspond to those shown in Figure 1. None of these brain regions responded to irradiation as determined by individual T-tests.

Figure 6. Uptake of 2-deoxyglucose into four brain regions in anesthetized rats which were either sham irradiated or microwave irradiated with the long axis of the animal's body parallel to either the electric (E||L) or propagation vector (K||L). Values represent means $\pm$ the standard error ($n=6$); the brain regions numbered herein correspond to those shown in Figure 1. None of these brain regions responded to irradiation as determined by individual T-tests.

Table I. Incorporation of 2-deoxyglucose into rostral spinal cord of alert or anesthetized rats which were either sham irradiated or exposed to 2450 MHz microwaves with the long axis of their bodies aligned with either the electric vector (E||L) or the propagation vector (K||L). Values represent means $\pm$ standard errors (n=6).

INCORPORATION OF 2-DEOXYGLUCOSE (DPM/mg)		
	ALERT	ANESTHETIZED
CONTROL	32.5 $\pm$ 5.3	29.7 $\pm$ 2.9
E L IRRADIATED	35.7 $\pm$ 7.0	29.5 $\pm$ 8.0
K L IRRADIATED	36.9 $\pm$ 2.0	33.8 $\pm$ 6.6

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indicated by the presence of a pair of peaks or valleys symmetrically distributed about the center. No such differences were observed at any given power density from 0-2 mW/cm².

A statistical comparison of the results obtained for the sham irradiated cultures and each of the three irradiated groups was carried out for all 48 regions sampled along the long axis of the waveguide. Only one P-value ≤ 0.05 (i.e., $P = 0.04$) and 13 P-values ≤ 0.10 were obtained in 144 comparisons. Due to the large number of comparisons, 14 P-values ≤ 0.10 would be expected in the absence of any effect. In all 14 cases, the matching region located at a equal distance on the other side of the midline was not significant; pooling of the results obtained in paired regions always resulted in P-values $\gg 0.10$. Analysis of the pooled data revealed that the average standard error of the difference between the irradiated and control cultures was $\pm 3.6\%$ at each power level; thus, an increase or decrease in calcium efflux of $\pm 8.8\%$ would have yielded a $P = 0.02$ ($df = 32$).

DISCUSSION

The techniques described herein appear to be sensitive enough to readily detect an alteration of calcium efflux by cell cultures prepared from chick cerebral hemispheres by modulated electromagnetic fields provided that the magnitude of the effect exceeds 8.8%. Studies by other investigators have reported that calcium efflux was either stimulated or inhibited by to extents considerably in excess of 8.8% (Bawin et al [1975] 20%, Bawin and Adey [1976] 12-15%, Bawin et al [1978] 21.5%, and Blackman et al [1982] 32%). Thus, our results seem to support those of Shelton and Merritt [1979] in that no effect of modulated electromagnetic fields was observed.

However, Blackman et al [1979] has pointed out that the effect observed by Bawin and Adey is sensitive to the precise experimental conditions employed and has mentioned that the apparent effect differed when 4 and 10 samples were irradiated. Since our experimental conditions are quite different from those employed by earlier investigators, it is possible that one or more of the following differences might be masking the effect on calcium efflux. First, we irradiated monolayer cultures of chick cerebral neurons and glia rather than intact hemispheres. Since preparation of cultures alters the geometry of the nerve fibers and the three dimensional relationship between the neurons and glia, this might be a significant factor, especially if one views the nerve fibers as serving as "antennas". Second, we irradiated with a modulated microwave carrier frequency [41.8 GHz] as this allowed us to utilize a technique developed in an earlier study on microwave bioeffects which is ideal for the study of power windows [Partlow et al, 1981]. Since both modulated ELF [Bawin and Adey, 1976] and VHF [Bawin et al, 1975 and 1978; Balckman et al, 1977 and 1979] frequencies alter calcium efflux, it is hoped that the carrier frequency might not be critical in determining the effect of modulated weak electromagnetic fields on calcium efflux. Third, the irradiation system employed in the present study [Partlow et al, 1981a] is radically different from others previously employed. These several variables make it impossible to definitely exclude the existence of an effect of modulated weak electromagnetic fields on calcium efflux from brain tissue under other conditions.

RESULTS

Efflux of ^{45}Ca from Prelabelled Cell Monolayers. Confluent monolayers of cerebral neurons and glia were prelabelled for 30 min with ^{45}Ca as described in Materials and Methods. The medium was rapidly removed and the coverslips bearing the cultures were removed from the bottom of the 35 mm culture dish, rinsed, and then placed in 5 ml of ^{45}Ca -free culture medium. The results shown in Figure 1 demonstrate that around 80% of the ^{45}Ca in the tissue is readily diffusible and that a maximum amount of ^{45}Ca has left the monolayer after approximately 10 minutes. Twenty minutes was allowed for efflux during real or sham irradiation in all later experiments because this period was obviously sufficient to allow all freely diffusible ^{45}Ca to leave the monolayer. In addition, 20 minutes was the irradiation period used by most earlier investigators.

Determination of the Distribution of ^{45}Ca in Monolayer Cultures. Following autoradiography, the optical densities of regions of a sham irradiated monolayer culture lying immediately above the waveguide were determined (Fig. 2, dotted line). This data set is typical for both irradiated and sham irradiated mixed cultures of neurons and glia in that the distribution of ^{45}Ca is not uniform. It was presumed that this nonuniformity was due to the tendency of these cells to aggregate rather than to form an even cell monolayer as was observed with BHK cells [Partlow et al, 1981a]. These aggregates occurred randomly at different points along the long axis of the waveguide and might make it very difficult to assess the effect of any treatment.

In order to correct for this nonuniformity, all monolayer cultures were stained using Coomassie blue following autoradiography and the region of the cell monolayer corresponding to the area over the open end of the waveguide was also scanned by microdensitometry (Fig. 2, solid line). Since Coomassie blue selectively stains cell proteins, the distribution of this stain is taken as a measure of the distribution of cells in the area over the open end of the waveguide. In the sham irradiated culture shown in Figure 2, a close correlation obviously exists between the distributions of ^{45}Ca and cell proteins along the long axis of the waveguide. As a result, the distribution of ^{45}Ca can be corrected for the uneven distribution of cells by dividing the relative optical density of the autoradiograph in any given region by the relative optical density of the stained monolayer in the corresponding region. The corrected distribution of ^{45}Ca is shown in Figure 3 for the same sham irradiated control culture. None of the individual values differ by more than $\pm 11\%$ from control. In subsequent experiments, eight duplicates were run for each condition in order to reduce the observed variability.

Lack of Effect of Irradiation on Calcium Efflux from Cerebral Monolayer Cultures. All cultures were either irradiated with $0-2 \text{ mW/cm}^2$ at 41.8 GHz with sinusoidal modulation at 0, 9, or 16 Hz or were handled identically but sham irradiated. The distribution of ^{45}Ca was corrected by staining each cell monolayer following autoradiography as described above. The corrected distribution of ^{45}Ca along the long axis of the waveguide does not appear to be different in the irradiated and the sham irradiated cultures. Since regions equidistant from the center of the long axis of the waveguide were exposed to the same power density (Table I), the existence of a power window would be

IRRADIATION CONDITIONS

Irradiation was carried out in a walk-in incubator into which the open ended waveguide extended. Culture dishes were positioned precisely on top of the waveguide using alignment markers on the dishes. A peristaltic pump recirculated Leibowitz L-15 medium in order to maintain the cell monolayer at a constant temperature of 37.2 °C. It was found that the chick cerebral cells were very sensitive to the mechanical pumping action of the system. Therefore, a new pumping system was designed in which 4 inlets and 4 outlets were situated alternately around the edge of the dish every 45°. Under these conditions, a much gentler pumping action was sufficient to maintain the temperature of the cells directly over the waveguide at 37.2°C as measured by use of liquid crystal sheets [Partlow et al, 1981a].

CELL CULTURE

Preparation of Mixed Cultures. Chick cerebral brain cell cultures were prepared from 9-10 day chick embryos as previously described [Hanson et al, 1982]. Monolayer cultures were established by plating 2×10^6 cells in 0.5 ml on a polylysine-coated polystyrene coverslip. This coverslip was attached to the bottom of a 35 mm Falcon tissue culture dish in such a way as to create a culture depression 20 mm in diameter and 1 mm deep [Partlow et al, 1981a]. Cultures were grown in Leibowitz L-15 medium containing 10% fetal calf serum. All cultures were grown to confluency before irradiation.

Handling of Monolayer Cultures Treated with ^{45}Ca . The cultures were prelabelled with ^{45}Ca (Amersham CES.3) in tissue culture medium (10 uCi/ml) for 30 minutes at 37.2°C. The ^{45}Ca -containing medium was then removed and two 2 ml aliquots of culture medium lacking ^{45}Ca were then used to briefly rinse the monolayer. Four ml of L-15 culture medium lacking ^{45}Ca was then added to each dish and the system was connected to the pumping equipment which contained an additional 15 ml of L-15 lacking ^{45}Ca . The dish was placed on the open end of the waveguide, pumping was initiated, and irradiation was carried out for 20 min. Dishes were then removed and coverslips bearing the cell monolayers were detached from the 35 mm dishes, dipped three times in an isotonic sodium cacodylate buffer containing 0.01 M CaCl_2 , and briefly fixed by immersion in the same buffer containing 2% glutaraldehyde and 2% paraformaldehyde. Following fixation, the monolayers were air dried for preparation of autoradiographs.

Autoradiography was carried out as previously described [Partlow et al, 1981a] except that Kodak No-Screen X-Ray film was used. The optical density of this film increases linearly with the amount of radioactive material [Partlow et al, 1981b]. The autoradiographs were exposed for 3 weeks and developed as specified by the manufacturer.

Microdensitometry was carried out as described in Partlow et al [1981a]. A 100 micron wide slit was advanced along the region of the autoradiogram corresponding to the long axis of the waveguide and the optical density was determined of each adjacent area so that the amount of calcium attached to cells at each point along the long axis of the waveguide (i.e., at different power densities) could be quantified. In this manner, the amount of efflux from the cerebral cells was measured by determining the amount of labelled calcium left in the cells rather than by measuring the amount released. Thus, increased efflux in the irradiated portion of the cell monolayer would be indicated by a decrease in the density of the autoradiogram in that region.

ease of prelabelling with ^{45}Ca . In addition, if an effect was observed with mixed cultures, purified cultures of neurons or glia could readily be prepared to determine whether the effect on calcium efflux resulted from a change in one or both cell types. A microwave carrier frequency of 41.8 GHz was used because (1) irradiation equipment already existed which could be utilized, (2) earlier work with ELF and VHF carrier frequencies suggested that the carrier frequency was of secondary importance in determining the effect of a modulated electromagnetic field on calcium efflux, and (3) it allowed us to use a new in vitro technique developed in this laboratory for the analysis of microwave bioeffects [Partlow et al, 1981a].

This new procedure has the following advantages. First, it made it possible to directly expose a cell monolayer to a continuous gradient of microwave power which ranges from zero to the maximum for that irradiation system. Any adherent cell population is first grown on microwave-transparent coverslips and then irradiated by placing the culture dish directly on the open end of the microwave waveguide. Thus, the first microwave-absorbing material encountered by the radiation is the monolayer of cells covering the upper surface of the coverslip. Second, the procedure allows quantification of the heating (if any) produced in the cell monolayer by the irradiation by use of a temperature-sensitive liquid crystal sheet used in place of the coverslip. Third, local heating can be totally eliminated by use of a pumping system to recirculate the culture medium. Fourth, it allows the effects of a large number of different power densities to be determined in a single culture because of the cosine² power distribution which exists at the level of the cell monolayer immediately above the waveguide. Since power density varies along the long axis of the waveguide (0 at both edges and maximum in the center), one can assess the effects of different power levels by selectively examining groups of cells located at different positions along the long axis. Quantitative measurements are made by microdensitometry of an autoradiograph of the cell monolayer which reveals the distribution of ^{45}Ca .

A detailed analysis of data obtained from 33 irradiated (or sham irradiated) monolayer cultures of cells derived from chick cerebral hemispheres has demonstrated that microwave irradiation at 41.8 GHz with sinusoidal modulation at frequencies of 0, 9, or 16 Hz and at 24 power densities ranging from 0 to 2 mW/cm² did not affect calcium binding by mixed cultures of cerebral neurons and glia.

MATERIALS AND METHODS

MICROWAVE EQUIPMENT AND PROCEDURES

The microwave source was a klystron (OKI model 45V12) set at a frequency of 41.8 GHz and powered by a universal power supply (FXR model Z815B). The details of the irradiation procedure are given in an earlier paper (Partlow et al, 1981) except that the output from the klystron was sinusoidally modulated at 0 (continuous wave), 9, or 16 Hz. The maximum power density at the center of the waveguide was 2 mW/cm²; power density was determined as previously described [Partlow et al, 1981a].

An important paper by Bawin et al [1975] suggested one possible mechanism whereby modulated weak electric fields might be able to alter brain function. Calcium efflux from neonatal chick cerebral hemispheres prelabelled with labelled calcium was observed during exposure to 147 MHz sinusoidally modulated fields (0-35 Hz) for 20 min. Increased loss of calcium was observed at modulation frequencies between 6 and 20 Hz but not at either higher or lower frequencies. Thus, the concept of "frequency windows" was introduced.

A subsequent study by Bawin and Adey [1976] further explored the effects of weak sinusoidally modulated ELF fields on calcium efflux from neonatal chick cerebral hemispheres or selected regions of the adult cat CNS. Results obtained with chick brains suggested the presence of both frequency and power windows. Thus, maximum decreases in calcium efflux were only observed at modulation frequencies of 6 and 16 Hz and amplitudes of 10 and 56 V/M. Similarly, data on cat brain samples showed significantly decreased efflux only at 6 and 16 Hz and 56 V/M. Thus, modulated ELF fields appeared to decrease calcium efflux while modulated VHF fields were found to increase the same process.

Further information on the effects of modulated VHF fields was provided in a paper by Bawin et al [1978]. Exposure of chick cerebral hemispheres to 450 MHz fields modulated at 16 Hz was reported to result in an increase in calcium efflux at power levels of 0.1 and 1.0 mW/cm² but not at 0.05, 2.0, or 5.0 mW/cm². Thus, this study demonstrated that VHF fields, like ELF fields, appear to be subject to power window constraints. In addition, these observations support those previously made at another VHF frequency (147 MHz; Bawin et al [1975]) in that the exposure increased rather than decreased calcium release.

Partial replication of many of these basic observations has been provided by Blackman et al [1977]. These investigators examined the effects of 147 MHz fields modulated at 0-32 Hz and having power densities of 0-2 mW/cm² on calcium efflux from preloaded chick cerebral hemispheres. The observed increase in calcium efflux was broader than previously observed but still peaked at 16 Hz. In addition, the only power level which yielded significant results was 0.75 mW/cm². In more recent experiments conducted with rigorously paired controls, Blackman et al [1979] reported that the width of the power window varies with precise experimental conditions. Thus, when only 4 samples were simultaneously exposed, the window was exceedingly narrow (only 0.9 mW/cm²). In contrast, when 10 samples were simultaneously exposed, the window was quite broad (0.2 to 1.5 mW/cm²).

Shelton and Merritt [1979] have conducted a related experiment on the effects of microwave irradiation on calcium efflux from rat brain tissue. Irradiation was carried out at 1 GHz with square wave modulation at either 16 or 32 Hz and power densities of 0-15 mW/cm². No effects on calcium efflux were observed under any conditions examined. However, the authors correctly point out that their conditions differed from previously employed in several significant aspects (i.e., frequency of the carrier wave, species, and shape of modulating wave function).

In the present paper, we have examined the effects of modulated 41.8 GHz microwave irradiation on calcium efflux from monolayer cultures of embryonic chick neurons and glia derived from the cerebral hemispheres. Mixed monolayer cultures were used instead of entire chick cerebral hemispheres because of the

SUMMARY

Sq cm
A technique recently developed in this laboratory has been used to determine whether sinusoidally modulated (0, 9, or 16 Hz) microwave irradiation (41.8 GHz, 0-2 mW/cm²) can alter calcium efflux from brain tissues. Monolayer cultures of neurons and glia were prepared from the cerebral hemispheres of 9-10 day chick embryos and the cells were prelabelled with ⁴⁵Ca. All culture dishes were placed directly on the open end of a waveguide at 37.2°C and were either sham irradiated or irradiated for 20 min. Local heating was prevented by recirculation of the culture medium. Autoradiographs of the cell monolayers revealing the distribution of ⁴⁵Ca were quantified by microdensitometry. These distributions of ⁴⁵Ca were corrected for nonuniformity in the distribution of cells within the monolayer.

Since power density varies according to a cosine² relationship along the long axis of the waveguide, it was possible to assess the effect of the modulated weak microwaves at 24 power densities ranging from zero at each edge to 2 mW/cm² at the center of the waveguide. An analysis of calcium binding by microwave- and sham-irradiated cultures demonstrated that irradiation had no effect on calcium binding at any power density up to 2 mW/cm² and at any of the three modulating frequencies.

INTRODUCTION

Sq cm
Data gathered in two separate studies have supported the concept that electrical fields modulated at certain critical frequencies can alter the ongoing pattern of electrical activity in the CNS. Thus, Cavalas et al [1970] reported specific changes in the brain electrical activity of monkeys subjected to low level, low frequency electrical fields (7 Hz sine waves, 2.8 V peak-to-peak). Spectral analysis of the EEG in several specific brain regions after 3 or 4 hr of exposure revealed power peaks at 6-8 Hz. This suggested that the externally applied low power electric fields were in some way able to drive or enhance specific brain rhythms. Similar conclusions were reached by Bawin et al [1973]. In this study, cats were exposed to low intensity (1 mW/sq.cm. or less) very high frequency (VHF; 147 MHz) fields which were amplitude modulated at frequencies between 1 and 25 Hz. Results on unconditioned cats strongly suggested that naturally occurring EEG rhythms could be enhanced in selected brain regions by exposure to VHF fields modulated at the same frequency. Finally, data gathered by Takashima et al [1979] also demonstrated that weak electric fields can modify the brain's electrical activity. Rabbits chronically exposed for 2 hr each day for 6 weeks at 1-10 MHz (15 Hz modulation) at the level of 500-1000 V/M exhibited a decrease in high frequency components and an increase in low frequency components of the EEG. In contrast, exposure to lower field strengths (100-200 V/M) resulted in an abnormal EEG characterized by spindle-like signals with a frequency of 14-16 Hz [Takashima and Schwan, 1979]. Thus, a number of studies suggest that externally applied weak electric fields can affect brain function if the fields are modulated at biologically appropriate frequencies.

LACK OF AN EFFECT OF MODULATED MICROWAVE IRRADIATION ON
CALCIUM EFFLUX FROM MONOLAYER CULTURES OF CEREBRAL NEURONS AND GLIA

L. M. Partlow, L. J. Stensaas, L. G. Bush, D. W. Hill, and O. P. Gandhi,
Departments of Pharmacology (LMP and LGB), Physiology (LJS), and Electical
Engineering (OP), University of Utah, Salt Lake City, Utah 84132

FIGURE 6

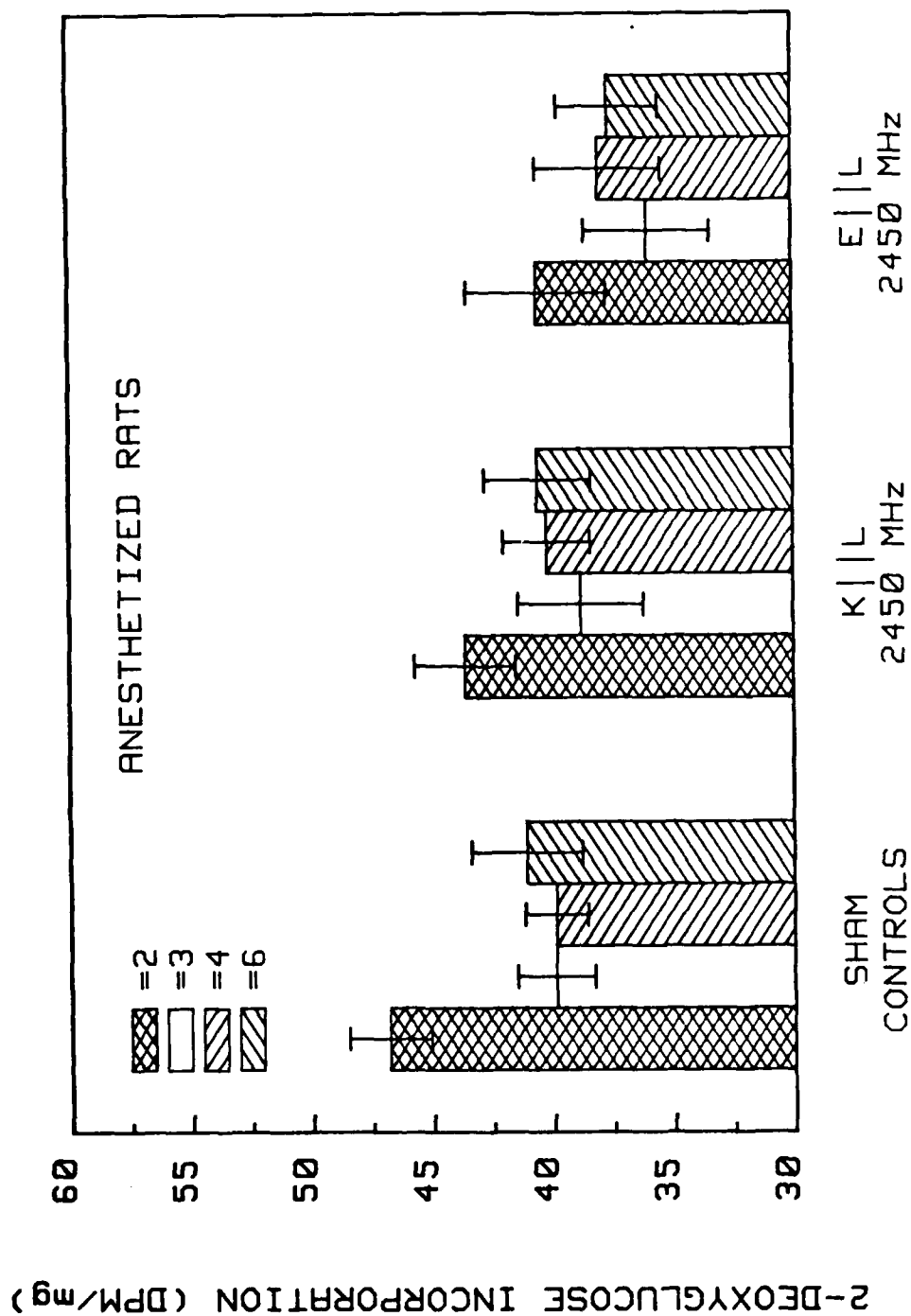


FIGURE 5

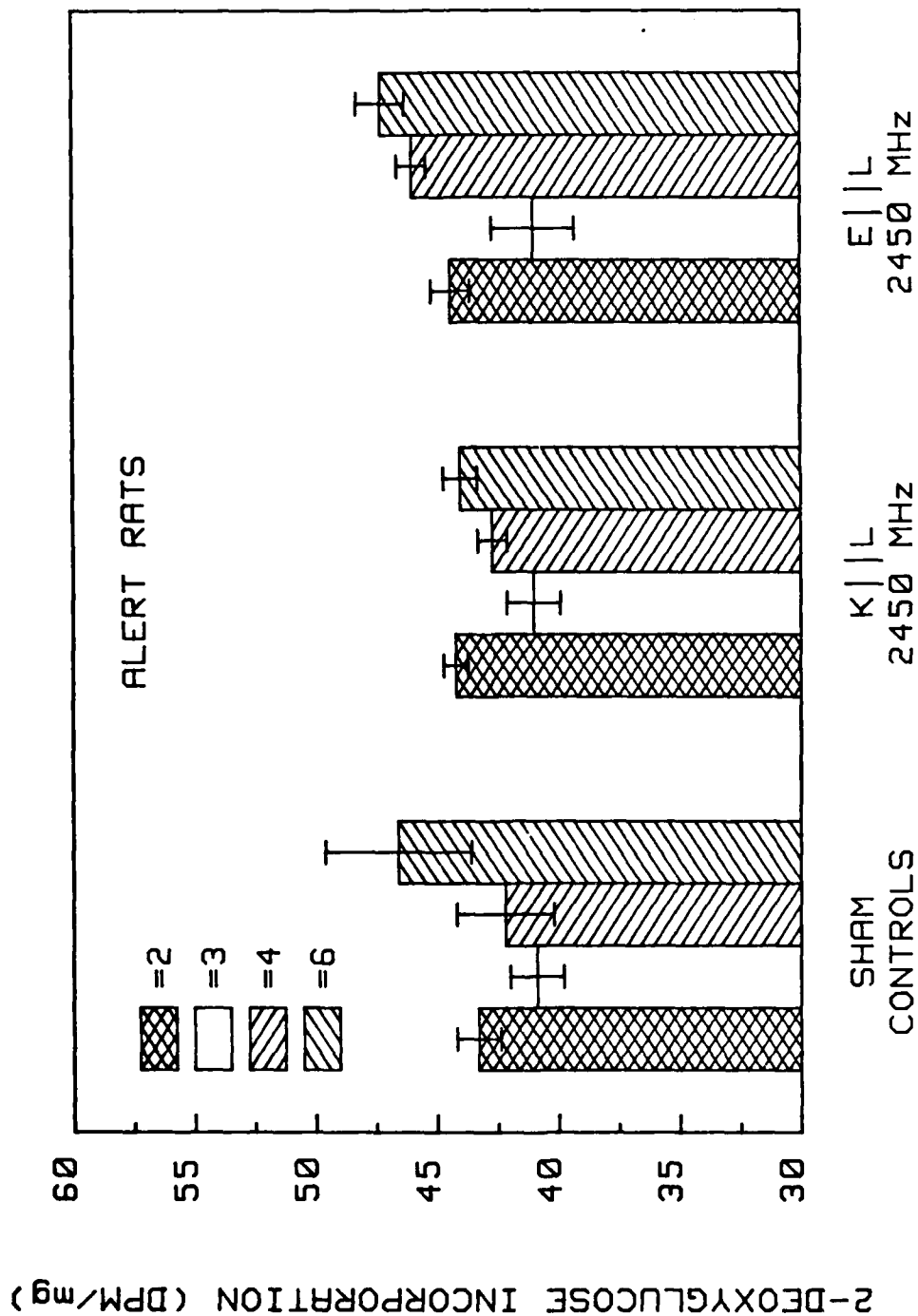


FIGURE 4

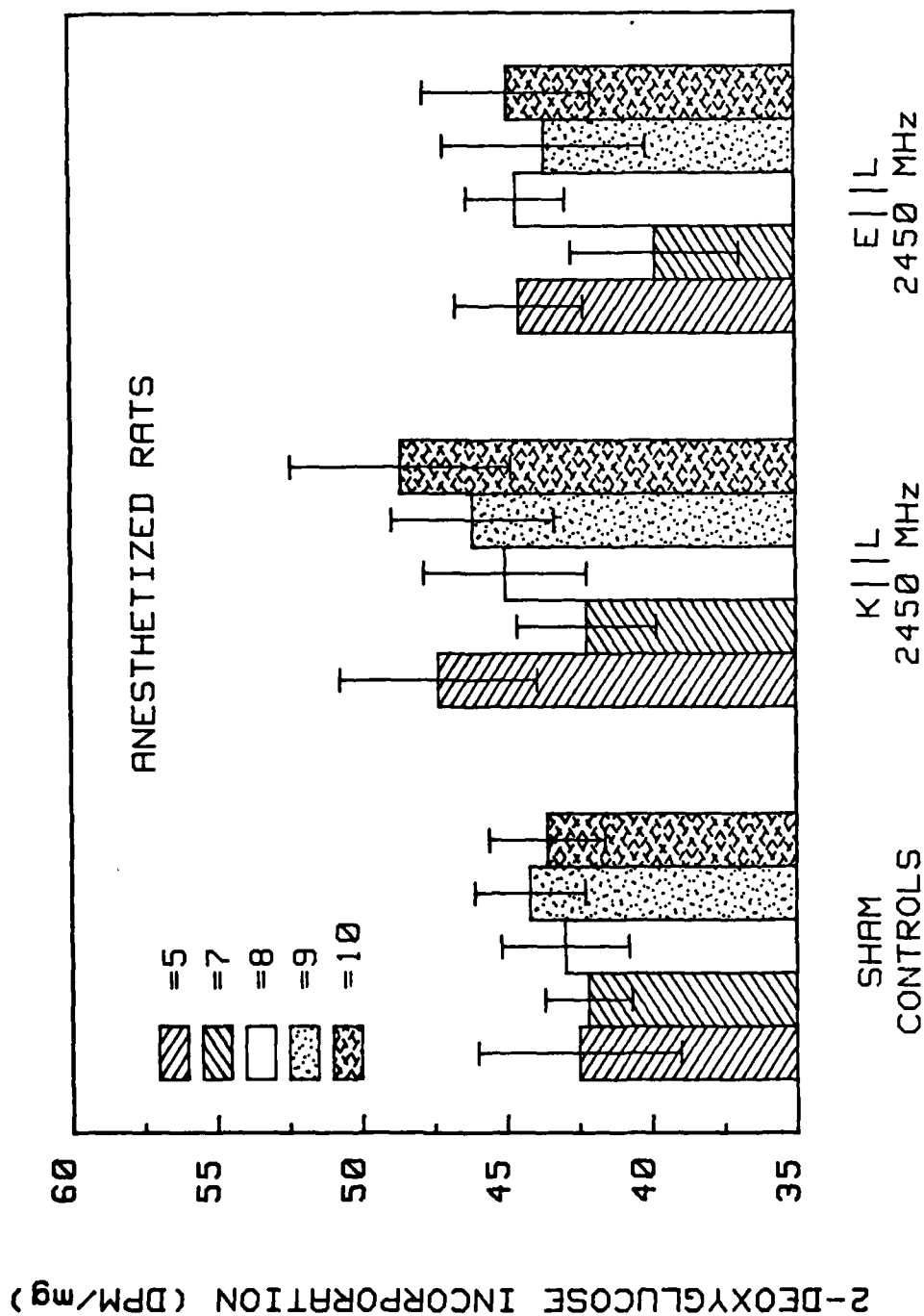


FIGURE 3

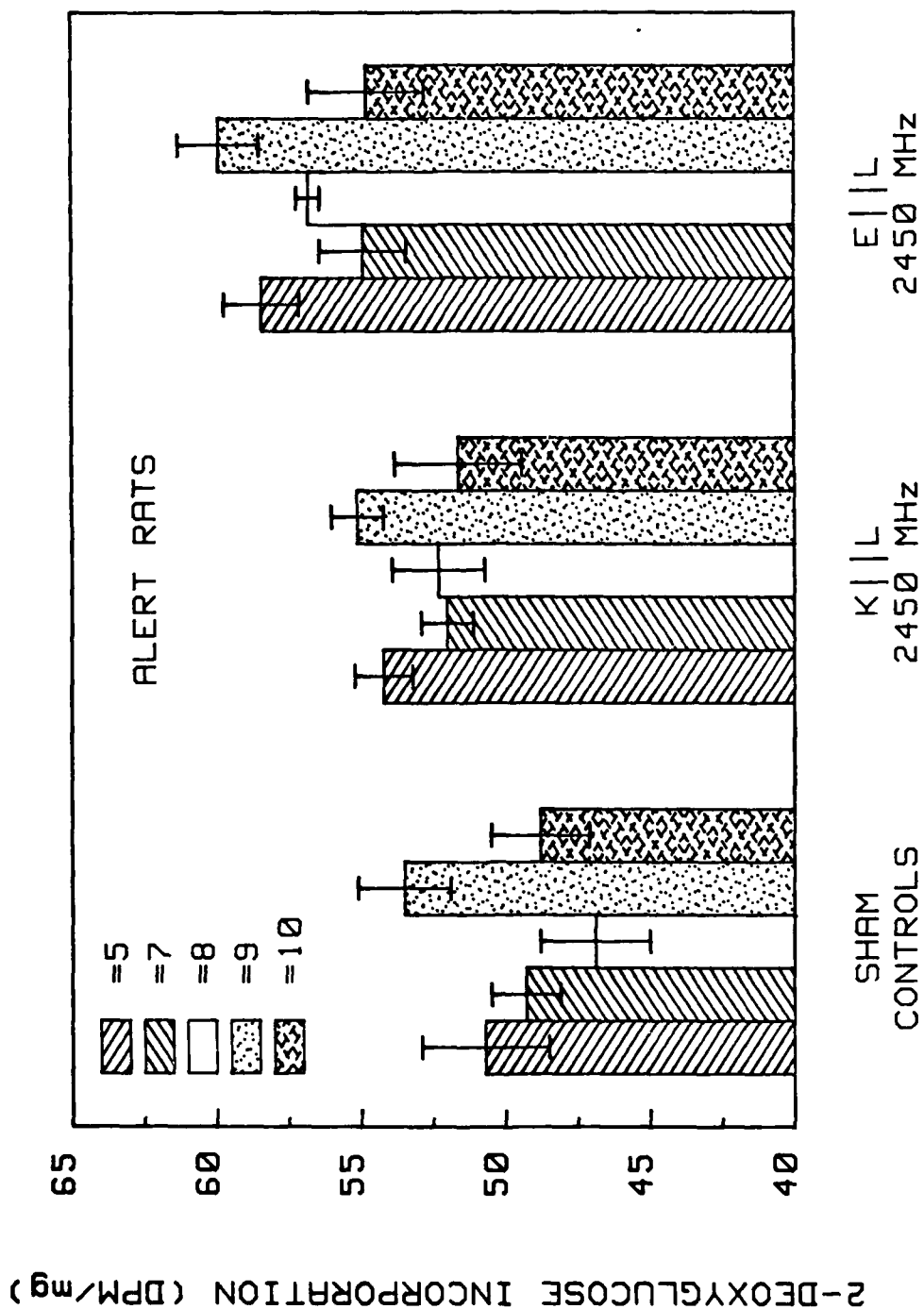


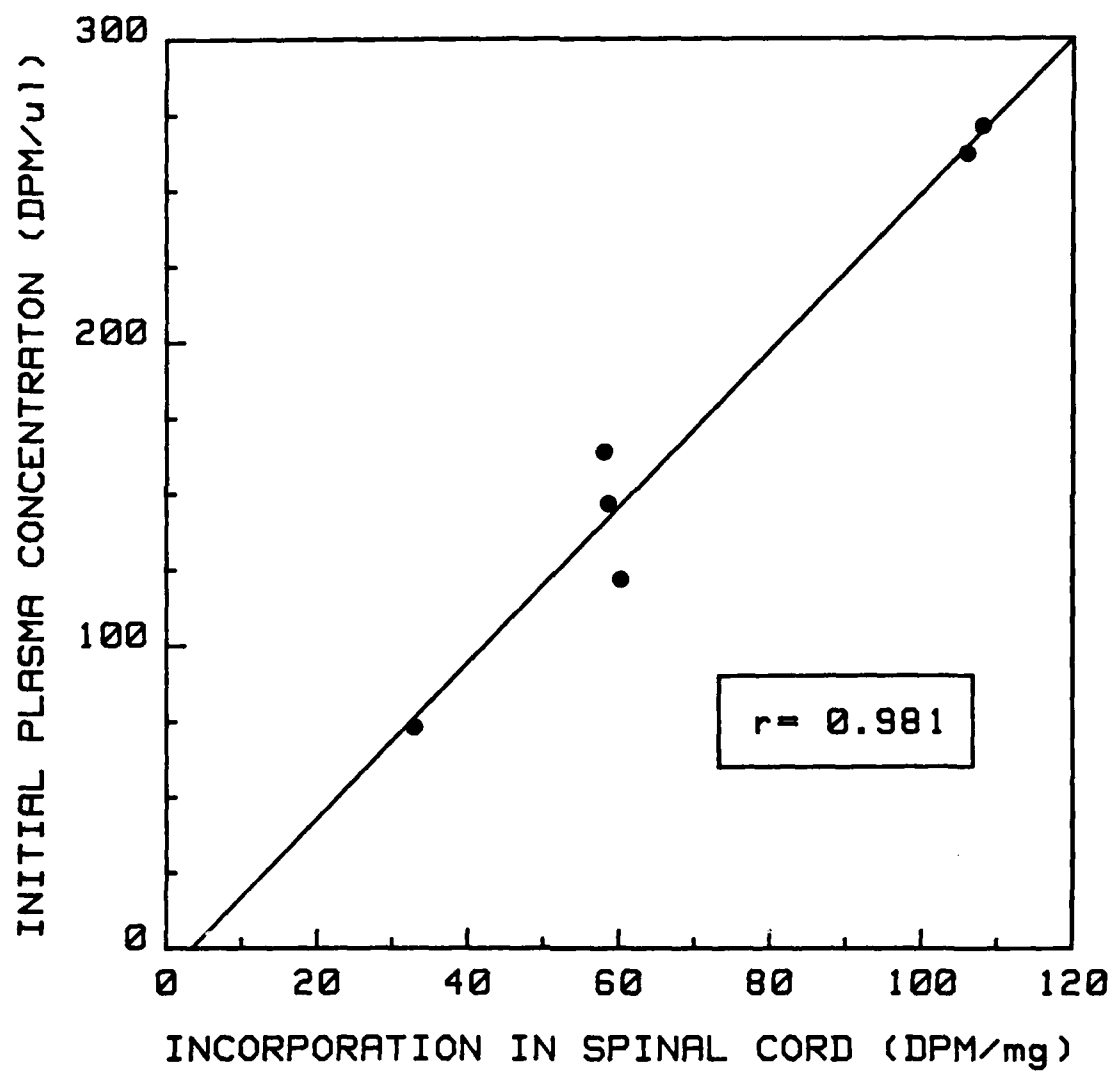
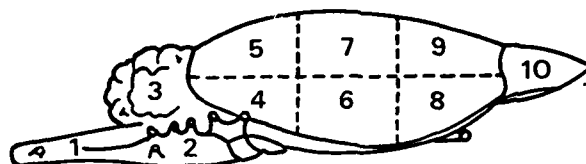
FIGURE 2

FIGURE 1



Shelton, W. W. and Merritt, J. H., In vitro study of microwave effects on calcium efflux in rat brain tissue. In: Abstracts of the 1979 URSI Meeting, June 18-22, Seattle, WA; pp.328, 1979.

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

Takashima, S., and Schwan, H. P., Effects of Radio Frequency Fields on the EEG of rabbit brains. In: Abstracts of the 1979 URSI Meeting, June 18-22, Seattle, WA; pp. 337, 1979.

Figure 1. Efflux of ^{45}Ca from prelabelled monolayer cultures of cerebral neurons and glia. All cultures were prepared, grown to confluency, and prelabelled as described in Materials and Methods. Coverslips bearing the monolayers were rapidly rinsed by dipping twice in culture medium and then immersed in 5 ml of culture medium. Aliquots from each bath [$n=4$] were taken at each time and the amount of ^{45}Ca remaining on the coverslip after 30 min was also determined.

Figure 2. Distribution of ^{45}Ca in different regions along the long axis of the waveguide in a sham irradiated monolayer culture [dotted line] as determined by measurement of the optical density of an autoradiograph of that culture. In addition, the distribution of cell proteins along the long axis of the waveguide was estimated for the same cerebral culture by measurement of the optical density of the coverslip bearing the monolayer culture following staining with Coomassie blue [solid line]. The culture was labelled, sham irradiated, and used for autoradiography as described in Materials and Methods. Following autoradiography, the cell monolayer was stained as previously described [Iversen et al, 1981]. Alignment marks on both the coverslip and the autoradiograph allowed examination of the region immediately above the waveguide in both cases.

Figure 3. Corrected distribution of ^{45}Ca in different regions along the long axis of the waveguide of a single sham irradiated monolayer cerebral culture. The corrected values were calculated for each region by determining the ratio of the relative optical densities of [1] the autoradiograph [dotted line, Fig. 2] and [2] the stained cell monolayer [solid line, Fig. 2].

Figure 4. Distribution of ^{45}Ca in different regions along the long axis of the waveguide in monolayer cerebral cultures which were either sham irradiated [A] or irradiated at an average power density of 1 mW/cm^2 with a carrier frequency of 41.8 GHz modulated at 0, 9, or 16 Hz [B, C, or D, respectively]. The mean and standard error are given for the relative optical density for 48 different regions [$n=9$ for A and $n=8$ for B, C, and D]. Since the power density distribution immediately above the open end of the waveguide is $\cos^2$, regions located at equal distances from the center of the waveguide were irradiated with equal power density; thus, the 48 regions represent pairs of areas exposed to 24 different power levels ranging from 0 to 2 mW/cm^2 . Statistical comparisons were made by comparing the results obtained with sham irradiated controls with those obtained for irradiated cultures.

Table I. Average power density impinging on regions of the cell monolayer immediately above the open end of the waveguide tabulated as a function of distance along the long axis from the center of the waveguide. These values were calculated using [1] the average power density of 1 mW/cm^2 which was obtained by initial equipment adjustment and [2] the cosine² distribution of power density along the long axis of the waveguide which has been shown to occur immediately above the open end of the waveguide [Partlow et al, 1981a].

<u>DISTANCE</u> (mm)	<u>POWER DENSITY</u> (mW/cm ²)
0.10	1.991
0.20	1.966
0.30	1.923
0.40	1.865
0.50	1.792
0.60	1.705
0.70	1.606
0.80	1.496
0.90	1.378
1.00	1.254
1.10	1.125
1.20	0.993
1.30	0.862
1.40	0.734
1.50	0.610
1.60	0.492
1.70	0.384
1.80	0.286
1.90	0.200
2.00	0.129
2.10	0.072
2.20	0.031
2.30	0.007
2.40	0.000

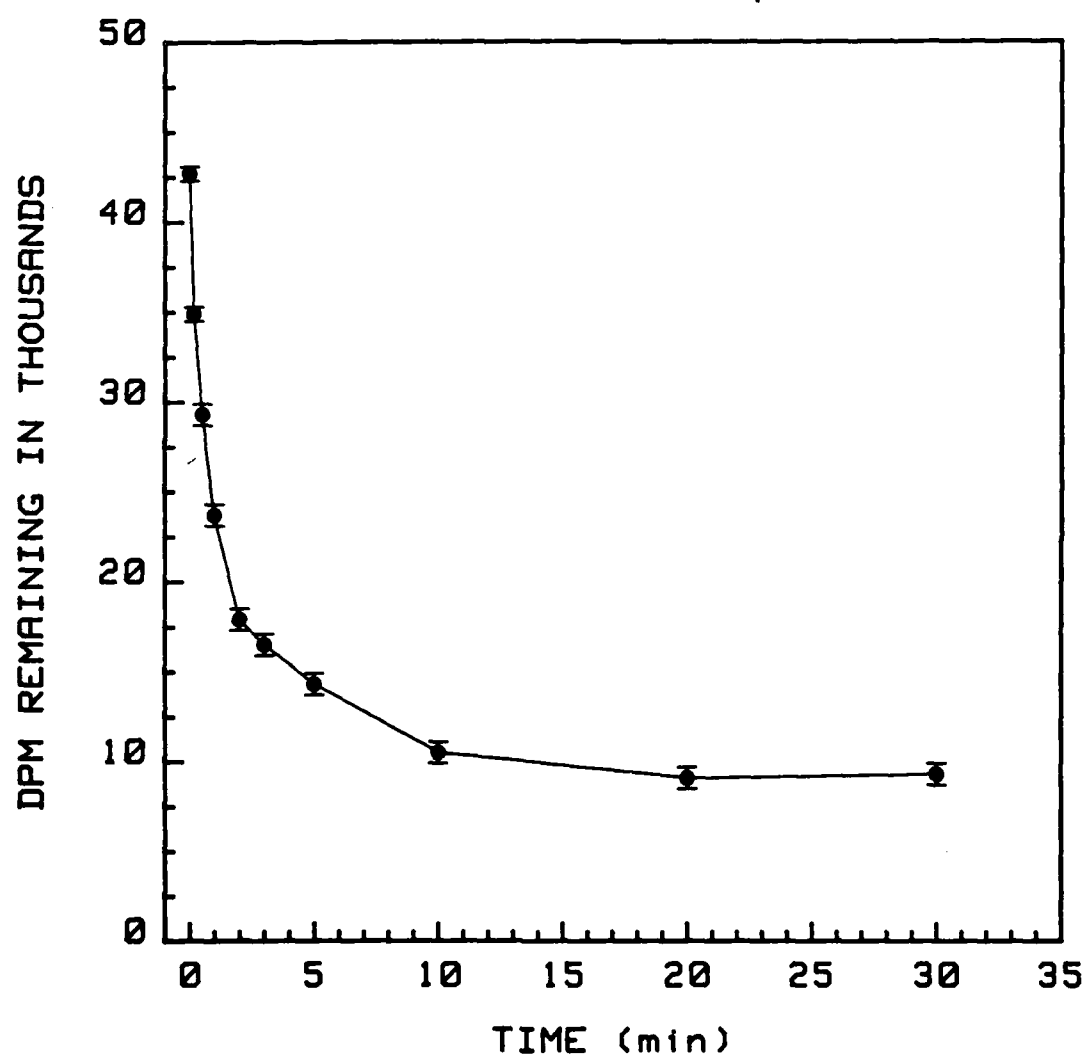
FIGURE 1.

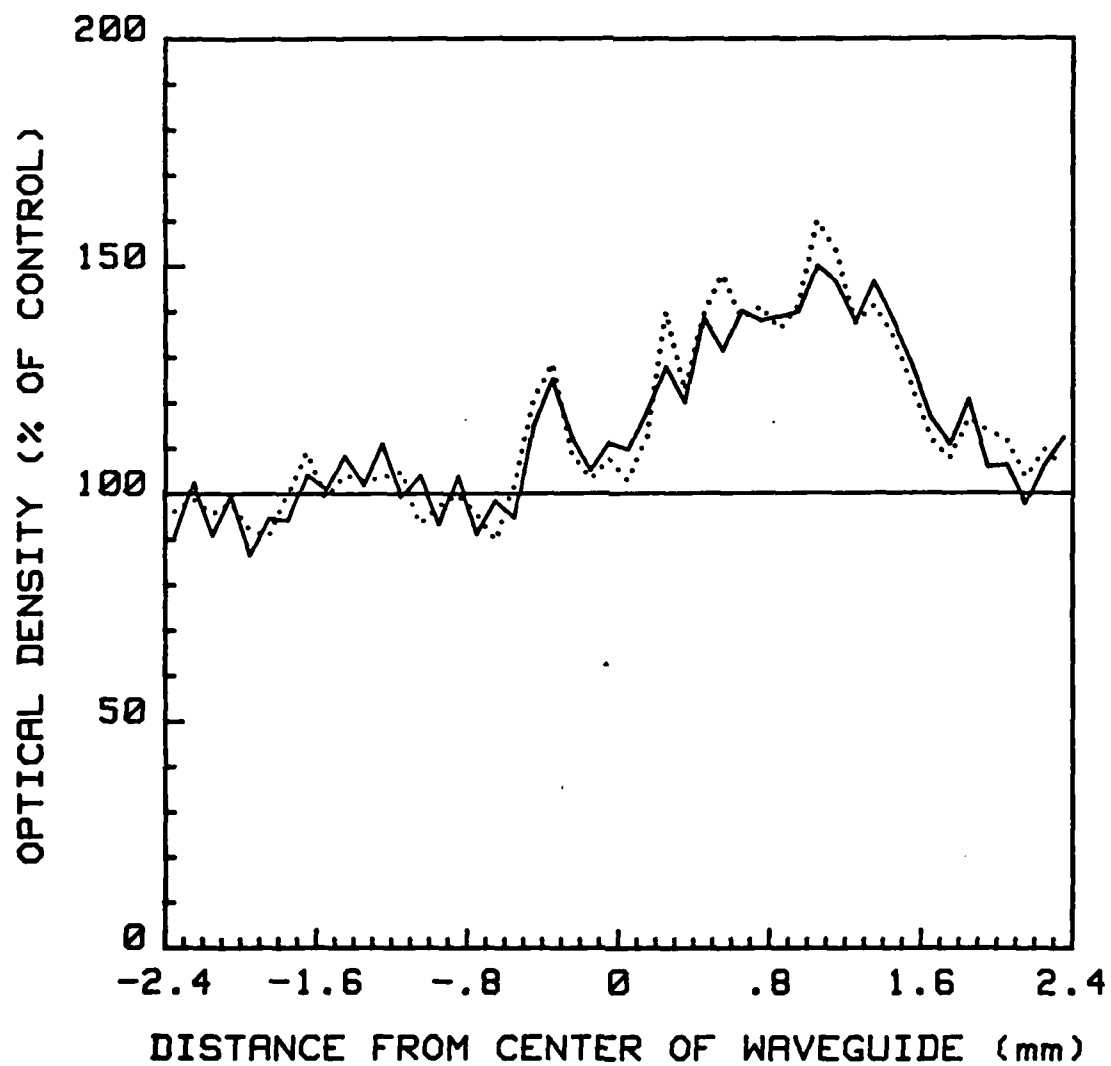
FIGURE 2.

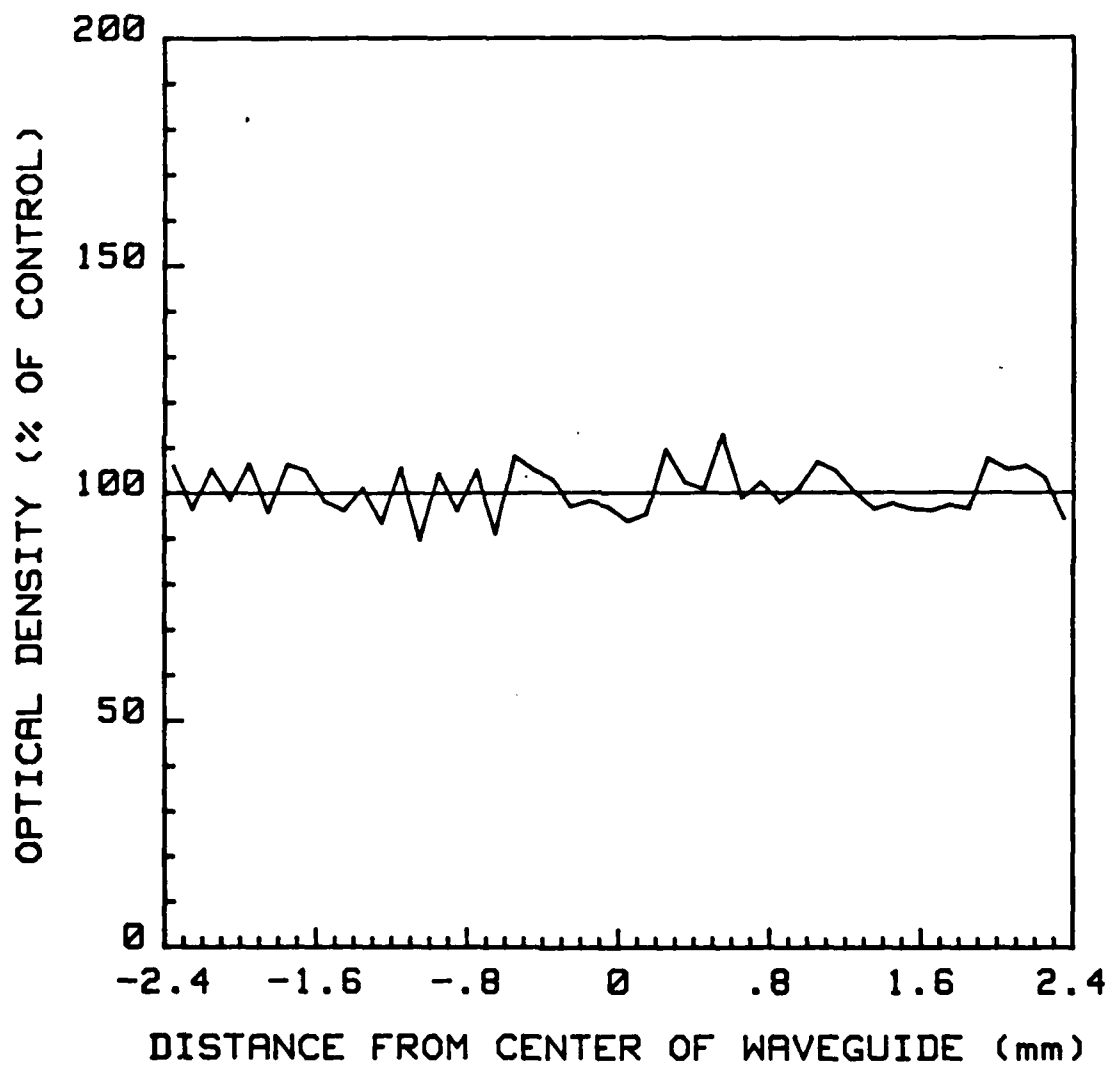
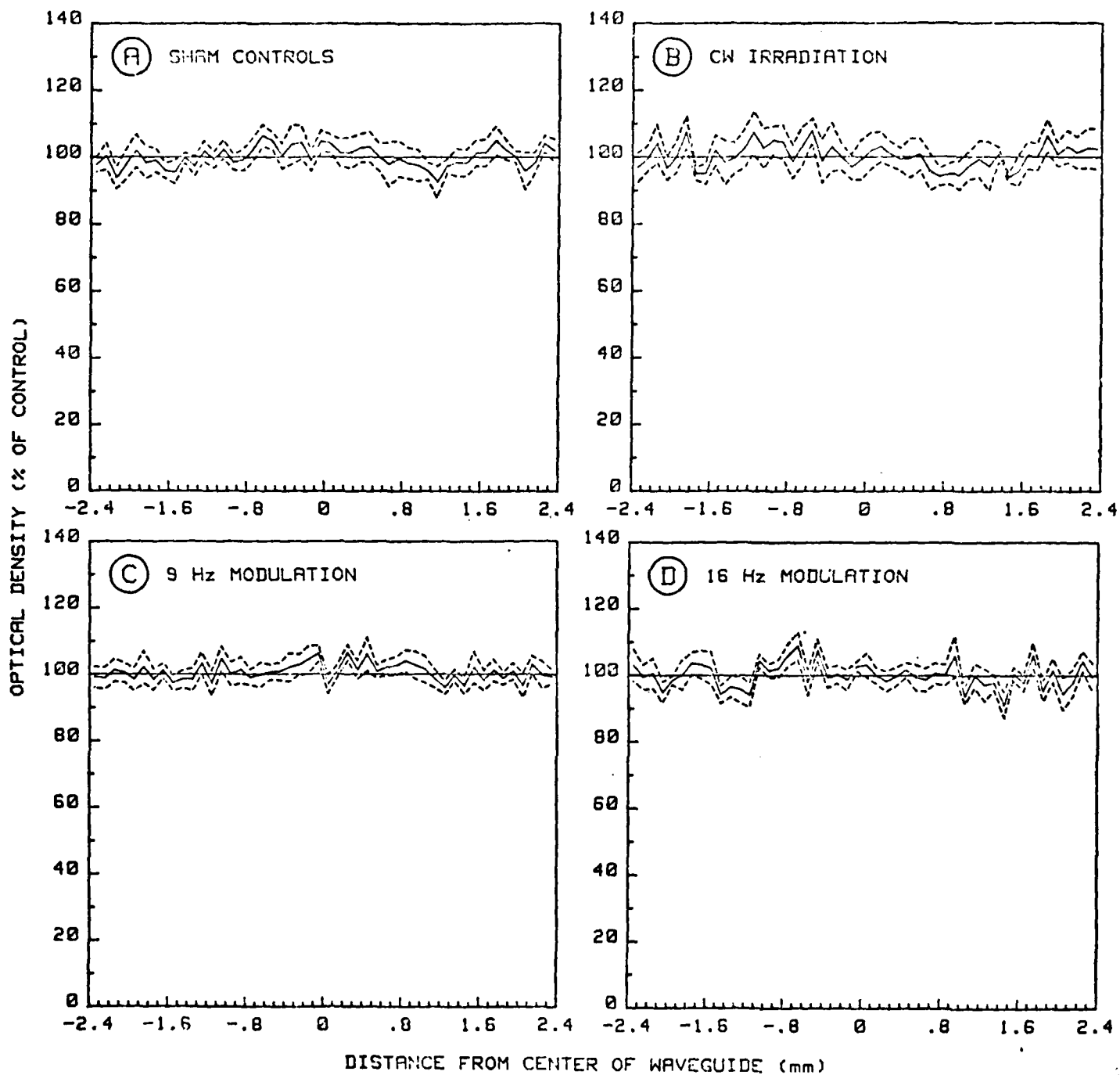
FIGURE 3.

FIGURE 4.

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