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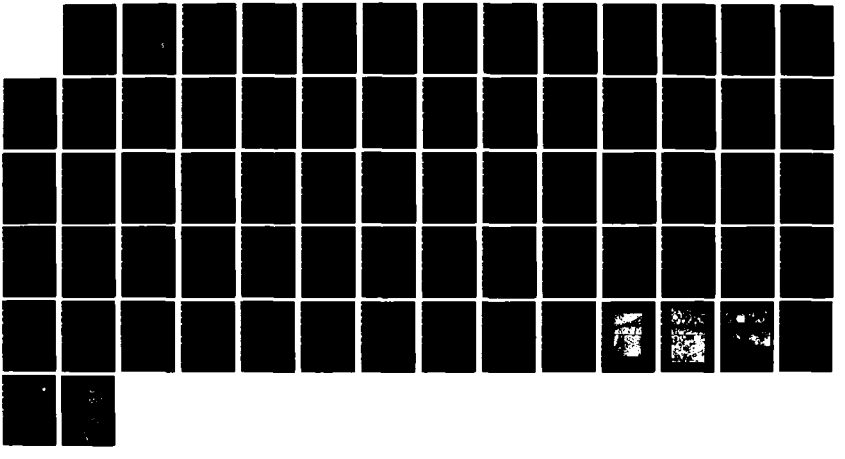

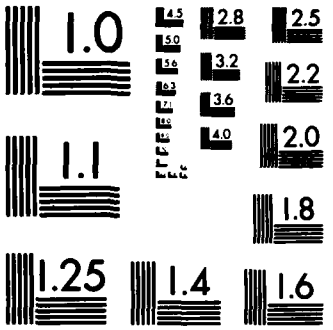

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FINAL REPORT

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MICROWAVE EFFECTS ON CNS
A Histopathologic, ultrastructural and Autoradiographic Study

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OBJECTIVES

The objectives of this investigation were to:

(a) investigate the effects of electromagnetic fields on the release of calcium ions from nervous tissue,

b) examine for histological changes, if any, of young rat brains after 2.45 GHz microwave exposure, and

c) study the rat brain metabolism during 2.45 GHz microwave exposure using 2-Deoxyglucose.

Objective a.

In order to meet this objective, two different experiments were conducted. The first experiment was designed to study the effects of 147 MHz, sinusoidally amplitude modulated at 16 Hz, radiofrequency on chick brain tissue slices. The second experiment was conducted under the same exposure parameters except chick cerebral hemispheres, instead of brain slices were used.

For experiment one, chick forebrains were removed from 1-6 day-old birds and quickly immersed in modified Krebs-Henseleit bicarbonate saline at room temperature. The medium was freshly gassed with 5% carbon dioxide: 95% oxygen prior to immersion of the chick forebrain. The forebrain was cut coronally with a thin razor blade into 1-2 mm thick slices, which were then sliced cross-sectionally to separate grey from white matter. The separated, grey matter slices were finally cut into slices with dimensions of 1-2 mm. The brain tissue was irrigated 2-3 times with freshly gassed medium during preparation of the slices to maintain a medium pH of 7.4.

The cerebral cortex tissue slices from each forebrain were then transferred into 2.0 ml freshly gassed medium containing 0.5 micro-Curies $^{45}Ca^{++}$ per ml and incubated under a 5% carbon dioxide: 95% oxygen atmosphere in a 37°C water bath for 30 min. The radiolabelling medium was pipetted off and the tissue slices quickly washed twice with 5.0 ml medium. The tissue slices were then transferred into eight polystyrene culture tubes; 1.0 ml medium was added to each tube (containing 1 or 2 tissue slices) and the tube capped with a rubber stopper after 10-15 sec. gassing with 5% carbon dioxide: 95% oxygen. A set of four tubes was placed in each of two styrofoam supports; one support was placed inside the test chamber and the other support inside the control chamber.

After actual exposure of the tissue slices in the test chamber for 20 min. coincident with co-incubation at 37°C of the tissue slices in the control chamber, an 0.2 ml aliquot was removed from each tube for radioactivity determination. Upon removing the remainder of the medium, the 1 or 2 tissue slices in each tube were homogenized with a Teflon/glass homogenizer in 2.0 ml 1.0 N NaOH; aliquots of the homogenate were removed for radioactivity determination and determination of protein content according to the procedure of Lowry et al. [1]

For the second experiment, chick forebrains were immersed in a medium at room temperature and divided along the longitudinal fissure with a thin razor blade. The composition of this medium was 155 mM NaCl, 5.6 mM KCl, 2.16 mM $CaCl_2$, 2.4 mM $NaHCO_3$ and 11.1 mM D-glucose; this composition is identical to that of the medium employed by Adey [2,3,4] and Blackman [5,6,7] and their colleagues for preparing chick cerebral hemispheres and bathing them during exposure to 147 MHz radiofrequency

radiation. Each cerebral hemisphere was weighed and transferred into a polystyrene culture tube containing 1.0 ml medium with 0.5 microCuries $^{45}Ca^{++}$ per ml. After 30 min. incubation in a 37°C water bath, the radiolabelling medium was pipetted off and 2.0 ml medium was added to the tube. The tube was inverted into a 10 cm. square gauze pad, and the retained cerebral hemisphere quickly washed twice with 250 ml of the medium. Each hemisphere was then transferred into a new polystyrene culture tube containing 1.0 medium. A set of four culture tubes, each with a ^{45}Ca-radiolabelled cerebral hemisphere, was placed in one styrofoam support, and another set of four tubes, each with a matching, radiolabelled cerebral hemisphere, was placed in a second styrofoam support. One support was placed inside the test chamber and the other support inside the control chamber.

After actual or sham exposure of the cerebral hemispheres in the test chamber for 20 min. coincident with co-incubation at 37°C of the cerebral hemispheres in the control chamber, an 0.2 ml aliquot was removed from each tube for radioactivity determination. Upon removing the remainder of the medium, the cerebral hemisphere was homogenized with a Teflon/glass homogenizer in 2.0 ml 0.2 M MaOH and 0.1% (w/v) soidum dodecyl sulfate; an aliquot of the homogenate was removed for radioactivity determination.

Analytical Methods: All radiolabelled media and suspensions were mixed with Aquasol-2 (New England Nuclear, Boston, Ma.) and radioactivity determined (with automatic compensation for differential quenching) in a Beckman liquid scintillation counter. Protein concentrations were determined by the method of Lowry et al[1] using bovine serum albumin as a standard.

Materials: Essential and non-essential amino acid mixtures were purchased from MA Bioproducts, Md.: L-glutamine, sodium pyruvate, sodium fumarate and bovine serum albumin from Sigma Chemical Co., St. Louis, Mo.,; and $^{45}CaCl_2$ from New England Nuclear.

RESULTS

The results of experiment one are presented in Table 1. Ca^{++} efflux from the tissue slices is expressed as (1) the percentage of total $^{45}Ca^{++}$ initially present in the slices that was released under exposed and control conditions and (2) the amount of $^{45}Ca^{++}$ released per ug tissue protein under exposed and control conditions. All expressions of Ca^{++} efflux under either exposed or control conditions are averaged from four determinations in each experiment. When, for each experiment, the average percentage value representative of the exposed condition is divided by the average percentage value representative of the control condition, we find the average $\pm$ standard deviation value of this ratio from the seven experiments to be 0.99 ± 0.10. Analysis of these seven ratio values by a Student's t-test indicates that there is no significant difference between percentages of $^{45}Ca^{++}$ efflux under exposed and control conditions. When, for each of the latter four experiments, the average cpm ^{45}Ca released/g tissue protein in the exposed condition is divided by the corresponding value representative of the control condition, we find the average $\pm$ standard deviation value of this ratio from the four experiments to be 0.94 ± 0.28. Analysis of these four ratio values by a Student's t-test indicated that there is also no significant difference between $^{45}Ca^{++}$ efflux under exposed and control conditions when such efflux is expressed relative to tissue protein.

The results of the second experiment are presented in Tables 2 and 3. Ca^{++} efflux from the cerebral hemispheres is expressed in Table 2 as the percentage of total $^{45}Ca^{++}$ initially present in the hemispheres that was released under exposed and control conditions, and in Table 3 as the amount of $^{45}Ca^{++}$ released /g tissue (wet weight) under exposed and control conditions. In each row of Tables 2 and 3, the form of expression of Ca^{++} efflux under exposed and control conditions pertains to a matching pair of hemispheres. When, for each row, the percentage value for the exposed condition is divided by that for the control condition, we find the average $\pm$ standard deviation value of this ratio from the 20 separate measurements to be 0.96 ± 0.19. Analysis of these 20 ratio values by a Student's t-test indicates that there is no significant difference between percentages of $^{45}Ca^{++}$ efflux under exposed and control conditions. When, for each row, the $^{45}Ca^{++}$ released /g tissue in the exposed condition is divided by the corresponding value representative of the control conditions, we find the average $\pm$ standard deviation value of this ratio from the 20 separate measurements to be 0.92 ± 0.26. Analysis of these 20 ratio values by a Student's t-test indicates that there is also no significant difference between $^{45}Ca^{++}$ efflux under exposed and control conditions when such efflux is expressed relative to tissue mass.

Table 4 represents the results of two experiments in which medium for experiment two was used. When the data is analyzed in a manner identical to that employed for the data in Tables 2 and 3, we find the average $\pm$ standard deviation value of the percentage ratio from the 8 separate measurements to be 1.02 ± 0.14, and that of the cpm/g tissue ratio to be 1.08 ± 0.25. Application of the Student's t-test indicates

that there is no significant difference between $^{45}Ca^{++}$ efflux under sham exposed and control conditions, independently of the manner in which such efflux is expressed.

DISCUSSION

The principal finding of this study is that there is no evidence to indicate that exposure of chick brain tissue to 147-MHz radiation, amplitude modulated at 16 Hz and applied at a power density of 0.75 mW/cm^2, significantly perturbs $^{45}Ca^{++}$ efflux. The significance of this negative evidence is that the data were secured from two distinct protocols for preparing and radiolabelling the chick brain tissue. It is, therefore, important to consider the strengths and weaknesses inherent in the experimental design of each protocol with respect to testing the effect of radiofrequency radiation on Ca^{++} efflux.

The primary advantage of the first experimental design is that the brain tissue is rapidly reduced, following euthanasia of the animal, to dimensions optimal for short-term in vitro support. Tissue slices with dimensions of 1-2 mm are of a size that permit passive diffusion of dissolved gases, nutrients and metabolic waste products into and out from the slices during the time periods required to radiolabel the cells and subject them to radiofrequency radiation. The secondary strengths are that (a) the tissue slices are incubated in a medium whose nutrient and dissolved gas composition can support the characteristic, high metabolic rate of nervous tissue cells and (b) all the tissue slices in each experiment are simultaneously radiolabelled with $^{45}Ca^{++}$ in the same

culture tube. The major disadvantage of the experiment is that multiple determinations of $^{45}Ca^{++}$ efflux from control and exposed slices must be made in each experiment and an averaged expression of $^{45}Ca^{++}$ efflux calculated from these determinations.

The procedures conducted for the preparation and radiolabelling of cerebral hemispheres in the second experiment reproduce as closely as possible those conducted by Adey[2,3,4] and Blackman[5,6,7] and their colleagues in their studies. Nonetheless, neither experiment yields data consistent with the argument that appropriately modulated 147 MHz radiation perturbs Ca^{++} efflux from chick brain tumors. This negative finding applies when $^{45}Ca^{++}$ efflux is expressed on a tissue mass basis, as Adey[2,3,4] and Blackman[5,6,7] have done, or on a percentage basis.

It is also of interest to note that application of the Student's t-test to the raw data in Table 2 (that is, analysis of the difference between the cpm released/g tissue data for the exposed condition with that for the control condition) shows that there is no significant difference between the two columns of data, which is the same conclusion drawn when similar analysis is applied to the 20 test/control ratio values. However, application of the Student's t-test to the inverted ratio values (that is, the control/test ratio values) yields a t-statistic of 2.37, a magnitude that suggests there is a significant difference at the 98% confidence level between the $^{45}Ca^{++}$ efflux under control and test conditions.

Blackman and his colleagues,[5,6,7] in particular, have conducted very extensive studies demonstrating enhanced Ca^{++} efflux during exposure to radiofrequency radiation with only highly specified carrier and amplitude modulation frequencies and power density ranges. In view of their reports, we believe that the most prudent interpretation of our findings

is that the exposure conditions we have used are not sufficienty by themselves to enhance Ca^{++} efflux. In other words, it is possible that Blackman and his colleagues report positive findings and we negative findings, using identical electromagnetic fields, because Blackman's group introduces specific factors (such as the spacing and number of culture tubes in the test chamber) in their experiments which complement the radiofrequency radiation sufficiently to enhance Ca^{++} efflux.[5,6,7] However, this possibility raises the issue that appropriate electromagnetic fields may or may not affect Ca^{++} fluxes in brain tissue depending upon the simultaneous presence of other physical factors separate from that of the non-ionizing radiation.

In conclusion, the data presented here demonstrates that the specific radiofrequency radiation used does not perturb Ca^{++} efflux from avian brain tissue under <u>in vitro</u> conditions. Comparison of this data with that gathered by Blackman's group, however, suggests that appropriate radiofrequency radiation in conjuction with certain other physical factors may indeed perturb Ca^{++} efflux.

LEGENDS

Table 1: Results of experiments in which chick brain tissue slices were prepared as in experiment 1 and ^{45}Ca efflux determined upon incubation in the control and test chambers, with the tissue slices in the test chamber being exposed to 147-Mhz radiation.

Tables 2 and 3: Results of experiments in which chick cerebral hemispheres were prepared as in experiment 2 and ^{45}Ca efflux determined upon incubation in the control and test chambers, with the cerebral hemispheres in the test chamber being exposed to 147-MHz radiation.

Table 4: Results of experiments in which chick cerebral hemispheres were prepared as in experiment 2 and ^{45}Ca efflux determined upon incubation in the control and test chambers, with the cerebral hemispheres in the test chamber being subject to sham exposure.

Table 1

Experiment	Percentage ^{45}Ca Released from Tissue Slices in Test Chamber	Percentage ^{45}Ca Released from Tissue Slices in Control Chamber	Test/ Control Ratio	cpm ^{45}Ca Released/µg Tissue Protein in Test Chamber	cpm ^{45}Ca Released/µg Tissue Protein in Control Chamber	Test/ Control Ratio
1	24.8 ± 3.1	23.0 ± 3.0	1.08			
2	15.4 ± 2.5	17.4 ± 4.7	0.89			
3	34.6 ± 2.1	32.2 ± 4.9	1.07			
4	45.6 ± 3.8	40.1 ± 0.7	1.14	0.97 ± 0.15	0.76 ± 0.29	1.28
5	38.3 ± 5.2	40.7 ± 8.7	0.94	0.80 ± 0.20	0.96 ± 0.36	0.83
6	26.4 ± 3.3	28.3 ± 4.8	0.93	0.82 ± 0.46	1.33 ± 0.94	0.62
7	24.1 ± 2.9	27.5 ± 2.1	0.88	0.67 ± 0.20	0.65 ± 0.08	1.03

Table 2

Experiment	Percentage ^{45}Ca Released from Cerebral Hemisphere in Test Chamber	Percentage ^{45}Ca Released from Cerebral Hemisphere in Control Chamber	Test/ Control Ratio
1	19.7	18.4	1.07
	23.8	24.2	0.98
	21.2	20.8	1.02
	18.6	20.2	0.92
2	18.0	24.6	0.73
	19.0	19.4	0.98
	18.6	23.0	0.81
	21.5	22.9	0.94
3	17.7	25.3	0.70
	22.4	22.0	1.02
	18.8	23.9	0.79
	19.4	16.5	1.18
4	24.6	16.8	1.46
	17.5	22.8	0.77
	18.2	20.2	0.90
	25.4	21.6	1.18
5	15.5	20.9	0.74
	17.5	17.2	1.02
	23.3	19.6	1.19
	23.6	28.6	0.83

Table 3

Experiment	cpm ^{45}Ca Released/gm Tissue in Test Chamber	cpm ^{45}Ca Released/gm Tissue in Control Chamber	Test/ Control Ratio
1	5827	6592	0.88
	7541	9958	0.76
	6854	7609	0.90
	7295	7109	1.03
2	5481	8563	0.64
	6217	5804	1.07
	4935	8217	0.60
	4792	7120	0.67
3	6192	11500	0.54
	7760	10522	0.74
	6981	8615	0.81
	5288	4423	1.20
4	8517	7400	1.15
	8940	8962	1.00
	4769	7120	0.67
	10017	8034	1.25
5	5774	8200	0.70
	7271	5442	1.34
	8117	5917	1.37
	7065	6929	1.02

Table 4

Experiment	Percentage ^{45}Ca Released from Cerebral Hemisphere in Sham Chamber	Percentage ^{45}Ca Released from Cerebral Hemisphere in Control Chamber	Sham/ Control Ratio	cpm ^{45}Ca Released/gm Tissue in Sham Chamber	cpm ^{45}Ca Released/gm Tissue in Control Chamber	Sham/ Control Ratio
1	16.4	19.5	0.84	6204	8364	0.74
	19.3	18.3	1.05	5583	6217	0.90
	16.5	15.9	1.04	7260	6129	1.18
	22.9	18.2	1.26	7074	5058	1.40
2	17.9	16.3	1.10	5162	5299	0.97
	15.6	17.7	0.88	3966	4369	0.91
	16.9	17.9	0.94	5172	4783	1.08
	16.8	16.7	1.01	6730	4560	1.48

Objective b.

Objective b was to examine for histological changes, if any, of young rat brains after 2.45 GHz microwave exposure. In order to meet this objective, newborn Sprague Dawley rats were exposed in plexiglas containers with internal dimensions of 19 x 11.4 x 7.6 cm. The tops and bottoms of these containers consisted of removable polystyrene grids to permit proper ventilation. Each container was positioned reproducibly on a plexiglas rack 208 cm. below a trunkated horn antenna. Sham-exposed animals were similarly housed in a separate anechoic chamber.

In an effort to define the exposure of individual subjects, a series of three field intensity measurements were made within each of the plexiglas containters. The measurements were made with and without non-experimental subjects in the remaining containers. All measurements were made with non-perturbing, three-dimensional probe (Collins S/N 57) and EIT digital receiver (S/N 1004).

It was determined that exposures varied as function of position within the container and also with time due to field perturbations produced by the movements of other animals. Consequently, although the average field intensity was 10 mW/cm^2, an animal might have received as little as 4 mW/cm^2 or as high as 30 mW/cm^2 at any instant. Utilizing the Radio-Frequency Radiation Dosimetry Handbook (Durney et al.),[8] specific absorption rate (SAR) for these rats was computed to be 2mW/g.

Matching sets of litter mates from both groups of animals, the one-day and six-day-old pups were placed in well-ventilated plexiglas cages in anechoic chambers (as described above) for 3.5 hours (9:00 a.m. - 12:30 p.m.). One chamber was energized so that the pups were irradiated with 2.45 Ghz (cw) microwaves at 10 mW/cm^2, while the other chamber was used for sham irradiation. After 3.5 hours of irradiation, the pups were

reunited with their mothers outside the anechoic chambers for 1.5 hours for feeding. At 2:00 p.m., control and experimental pups were again placed in their respective chambers and irradiated for an additional 3.5 hours (2:00 p.m. - 5:30 p.m.). The pups were then returned to the cages with their mothers until the next morning. This procedure was repeated daily for five days. Thus, the young rats were irradiated for 7 hours/day for five consecutive days. On the sixth day, all animals were anesthetized by pentobarbital sodium (50 mg/kg) and fixed by intracardiac perfusion. Animals used for light microscopy were perfused with 10% buffered formalin. Those for electron microscopy were perfused with weak Karnovsky's fixatvie containing 1% paraformaldehyde, 1.25% glutaraldehyde, 0.12% $CaCl_2$ in Cacodylate buffer. After perfusion, the brains were dissected out, the cerebella were separated and left in the fixatives for 12 hours.

For light microscopy, the cerebella were bisected in the median plane, dehydrated, double-imbedded in paraffin-celloidin and serially sectioned at 10 um in the sagittal plane. Sections were stained alternatively with hematoxylin and eosin and 1% thionin in Wolpole acette buffer at pH 4.8 for Nissl substance. The entire series of sections from experimental and control animals were matched and examined according to predetermined 5 parasagittal planes (Albert et al.).[9] This step was necessary to insure that identical areas of the cerebellar folia and EGL were examined in both experimental and control animals.

For electron microscopy, thin sagittal slices (1-2mm thick) were cut from the cerebella at the vermal and paravermal regions. Small wedges from the cortical area of matching folia were cleanly dissected and processed for electron microscopic examination.

Observations and Results:

I. Light microscopic examination of cerebella of irradiated (experimental and sham irradiated (control) animals revealed the following:

a) Along with mitotic cells, there were many small deeply stained cells scattered among the developing normal microneurons in the EGL of both experimental and control animals. However, there was a consistently greater preponderance of these cells in the experimental animals than in controls. The deeply stained cells possessed hyperchromatic pyknotic nuclei, usually rounded and centrally placed. In some cells, the nuclei were eccentric, indented or fragmented (See paper by Albert et al in Appendix.). These observations are indicative of various stages of degenerations. The degenerating cells were scattered throughout the EGL. These cells were counted in 3 serial parasagittal sections at 5 different matching planes of the cerebella of both experimental and control animals. The results indicated a statistically significant increase in the relative number of pyknotic cells per folium in the irradiated animals. Their number in the experimental animals was approximately twice as much as their number in the control animals. (Table 5).

b) In addition to the changes in the EGL cells, there was a change in the pattern of Nissl bodies of many Purkinje cells in experimental animals in comparison to controls. The Nissl bodies were in the form of finely dispersed granules instead of the compact rod-shaped appearance generally seen under the light microscope in normal Purkinje cells (See paper by Albert et al in Appendix.). However, typical chromatolytic patterns were not observed.

c) Some folia of the irradiated animals were studded with mononucleated blood cells, usually near a capillary blood vessel in the white core. The mononucleated cells were dispersed over the internal granular layer as well. Neither extravasation of red blood cells nor any pathological changes of the blood vessels were observed. It is noteworthy that the vascular cellular infiltration was not unique to that of any specific or non-specific inflammatory conditions.

II. Electron microscopic observations:

a) The pyknotic cells observed under light microscopy presented large rounded nuclei compactly packed with homogenous electron dense material (heterochromatin), concealing any nuclear details. Some of these cells presented ruptured nuclear membranes with leakage of the nuclear contents in a dense cytoplasm. Some cells showed extrusion of the nucleus with polarized compact heterochromatin and normal appearance of euchromatin. Others presented disintegrated nucleus with vacuolated cytoplam (See paper by Albert et al in Appendix.). This is interpreted as various stages of degeneration among the neuroblasts in EGL of irradiated animals. It was not uncommon to find the degenerated cells engulfed by other surrounding EGL cells, probably glioblasts.

b) On the other hand, most of the Purkinje cells of exposed animals mainly showed small, disorderly arrays of rough endoplasmic reticulum (RER) instead of the typical orderly stacks of parallel arrays (See paper by Albert et al in Appendix.). Nevertheless, such observations among Purkinje cells of irradiated animals is not suggestive of definite chromatolysis.

CONCLUSIONS

Light microscopy revealed that almost twice as many degenerating cells were seen in EGL of exposed animals over control. However, a very small percentage of the total EGL population was affected.

Electron microscopic evaluation suggests that the Nissl substance in Purkinje cells of exposed animals was more finely dispersed than that in cells of control animals.

These observations suggest that microwave irradiation may interfere with early genesis of cerebellar microneurons and alter the metabolic status of Purkinje cells. However, the real physiological significance of these changes in not clear and should be explored.

A manuscript submitted for publication is appended.

Effect of 2450 MHz (CW) microwaves at 10 mW/cm^2 on the external granular layer (EGL) of rat cerebella irradiated during day 1 - 5 and 6 - 10 postnatally*.

		Total No. of degenerated cells/ parasagittal section.	Relative No. of degenerated cells/folia in parasagittal section.	Difference between relative No. of degenerated cells in experimental and control cerebella.	Proportion of increase of relative No. of degenerated cells in experimental animals.
Animals irradiated day 1-5.	Experimental	52.1 SD±24.78	5.17 SD±1.89	2.22 SED±1.62	1:1.75
	Control	27.78 SD±10.5	2.95 SD±0.81	0.001<P<0.01	
Animals irradiated day 6-10.	Experimental	128.10 SD±35.01	11.76 SD±3.83	5.23 SED±3.11	1:1.80
	Control	76.14 SD±19.7	6.53 SD±1.71	0.02<p<0.05	

* All values are the mean number of degenerated cells counted in parasagittal sections at different planes studied in each group.

Table 5

Objective c.

Objective c was to test brain metabolism as revealed by labelled 2-Deoxyglucose uptake after microwave irradiation. The use of 2-Deoxyglucose for studying brain metabolism has been demonstrated to be a very effective tool for studying subtle changes. These include metabolic changes during anesthesia, sleep, temperature, and others.

This study is based on the principle that 2-Deoxyglucose is an analogue of glucose and reflects glucose comsumption of the brain. The advantage of using 2-Deoxyglucose is that unlike glucose, it can be trapped in the brain and does not metabolize to CO_2 and H_2O. Thus, one can measure trapped 2-Deoxyglucose in the brain tissue.

Adult rats injected with 2-Deoxyglucose were exposed to 10 mW/cm^2 (2800 MHz) for two hours. Another group of matching animals were also injected with 2-Deoxyglucose and were sham exposed. All animals were decapitated immediately following irradiation and the brains dissected and frozed in liquid nitrogen. These procedures were carried out at the Naval Medical Research Institute. The frozen brains were brought to our laboratory and then serially sectioned on a cryostat and prepared for autoradiography.

The results indicate that there was no substantial difference between control and irradiated animals. There were regional differences in the consumption of 2-Deoxyglucose but these regional differences were observed in controls as well as experimentals. Special attention was given to the hearing centers due to the auditory effects of microwaves. However, we could not discern any increased uptake of 2-Deoxyglucose in the white matter or the grey matter of the auditory system (cochlear nuclei, nucleus of the trapezoid body, superior olivary comples, ventro-

caudal nucleus of lateral lemniscus, inferior colliculus and the medial geniculate body).

It is concluded that exposure to 2800 MHz microwave radiation at 10 mW/cm^2 did not alter the metabolism of brain as measured by 2-Deoxyglucose. Further, it suggests that under these parameters, the brain temperature did not rise sufficiently to reflect greater uptake of 2-Deoxyglucose.

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APPENDIX

EFFECT OF AMPLITUDE-MODULATED 147MHz RADIOFREQUENCY RADIATION OF CALCIUM ION EFFLUX FROM AVIAN BRAIN TISSUE

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ABSTRACT

Cerebral cortex tissue slices and cerebral hemispheres prepared from Gallus domesticus chicks were exposed to 147-MHz radiofrequency radiation, amplitude modulated at 16 Hz and applied at a power density of 0.75 mW/cm^2, to determine the effect of such exposure on $^{45}Ca^{++}$ efflux from the avian brain tissue. Statistical analysis of these data demonstrates that such exposure has no significant effect on $^{45}Ca^{++}$ efflux.

INTRODUCTION

Calcium is a generalized regulatory signal of cellular functions. It serves this role in such diverse processes as muscle fiber contraction, microtubule assembly, stimulus-secretion coupling in glandular cells and hormone-mediated regulation of cyclic nucleotide levels. Regulation of Ca^{++} metabolism is also intimately associated with many nervous system functions. Ca^{++} couples excitation to neurotransmitter release, participates in ion influx during excitation and influences the generation of action potentials.

In recent years, Adey and Blackman and their colleagues have reported that exposure of chick cerebral hemispheres to sinusoidally amplitude-modulated, non-ionizing electromagnetic radiation can induce a statistically significant 10-18% increase in Ca^{++} efflux (1-6). Their studies have shown that the magnitude of this induction varies with the frequency of amplitude modulation and the power density of the incident radiation at specified carrier frequencies in the MHz range, and, furthermore, that the effective power density ranges are carrier frequency dependent. Correlation of this experimental data with theoretical predictions has led Joines and Blackman (7) to propose that the electric field intensity within the brain tissue, as opposed to thermal insult, is the primary determinant of the efflux effect. The results of these investigations have been extended in studies demonstrating direct effects of amplitude-modulated electromagnetic fields on calcium ion efflux from the awake cat

cerebral cortex (8), calcium ion efflux from synaptosome fractions (9), calcium ion-dependent activation of adenylate cyclase by parathyroid hormone in cultured bone cells (10), and calcium ion-dependent noradrenaline release from a clonal nerve cell line (11).

The impetus for the study reported here was to replicate the enhanced Ca^{++} efflux effect in an in vitro system designed to permit identification of which intracellular and/or extracellular Ca^{++} pools are perturbed by appropriate electromagnetic fields. The in vitro system consists of chick brain tissue slices incubated in a richly supplemented culture medium under a 5% carbon dioxide:95% oxygen atmosphere. Our inability to elicit the enhanced Ca^{++} efflux effect in this in vitro system prompted up to repeat the experiments with chick cerebral hemispheres. The findings presented here do not provide any evidence that exposure of avian brain tissue to 147-MHz radiation, amplitude modulated at 16 Hz and applied at a power density of 0.75 mW/cm^2, alters Ca^{++} efflux.

METHODS AND MATERIALS

Exposure Facilities: A 147-MHz signal, sinusoidally amplitude modulated at 16 Hz, was generated by a Hewlett-Packard (HP) 8660A synthesized signal generator coupled to an HP 86632B section and an HP86603A RF section; an HP 209A oscillator coupled to the modulation section provides the amplitude modulation signal. The signal was amplified (via an amplifier, Amplified Research, Souderton, Pa.; Model 10W1000) and transmitted through a bi-directional coaxial coupler (Narda Microwave Corp., Plainview, N.Y.; Model 3020A) before being transmitted through a Crawford Cell TEM test chamber (Instruments for Industry, Farmingdale, N.Y.; Model IFI110). In all experiments, actual exposure to the amplitude-modulated 147 MHz radiation was at a power density of 0.75 mW/cm^2. Measurements of power reflected from and transmitted through the test chamber demonstrated that (to within the sensitivity of the HP 432A power meters) all of the incident power was transmitted through the test chamber under all experimental conditions. The test chamber and a control chamber (consisting of a cylindrical metal container) were maintained at 37°C. during experimental runs by being housed in an incubator (Leahy Manufacturing Co., Higginsville, Mo.).

Preparation and Exposure of Avian Brain Tissue: Two different protocols were employed in preparing chick brain tissue for actual or sham exposure to 147 MHz radiofrequency radiation.

Protocol I: In experiments using Protocol I, Gallus domesticus chicks, one to six days old, were sacrificed by decapitation and the forebrain quickly immersed in modified Krebs-Henseleit bicarbonate saline (medium I) at room temperature (19-21°C.). The composition of medium I is 105.4 mM NaCl, 4.8 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 5.6 mM D-glucose, 2.0 mM L-glutamine, 4.9 mM sodium pyruvate, 5.4 mM sodium fumarate, 10 µg phenol red per liter, and essential and non-essential amino acids at concentrations as specified by Eagle (12). Medium I (with a pH of 7.4) was freshly gassed with 5% carbon dioxide: 95% oxygen prior to immersion of the chick forebrain. The forebrain was cut coronally with a thin razor blade into 1-2 mm thick slices, which were then sliced cross-sectionally to separate grey from white matter. The separated, grey matter slices were finally cut into slices with dimensions of 1-2 mm. The brain tissue was irrigated 2-3 times with freshly gassed medium I during preparation of the slices to maintain a medium pH of 7.4.

The cerebral cortex tissue slices from each forebrain were then transferred into 2.0 ml freshly gassed medium I containing 0.5 microCuries $^{45}Ca^{++}$ per ml and incubated under a 5% carbon dioxide: 95% oxygen atmosphere in a 37°C. water bath for 30 min. The radiolabelling medium was pipetted off and the tissue slices quickly washed twice with 5.0 ml medium I. The tissue slices were then transferred into eight polystyrene culture tubes; 1.0 ml medium I was added to each tube (containing 1 or 2 tissue slices) and the tube capped with a rubber stopper after 10-15 sec. gassing with 5% carbon dioxide: 95% oxygen. A set of four tubes was placed in each of two

styrofoam supports; one support was placed inside the test chamber and the other support inside the control chamber.

After actual exposure of the tissue slices in the test chamber for 20 min. coincident with co-incubation at 37°C. of the tissue slices in the control chamber, an 0.2 ml aliquot was removed from each tube for radioactivity determination. Upon removing the remainder of the medium, the 1 or 2 tissue slices in each tube were homogenized with a Teflon/glass homogenizer in 2.0 ml 1.0 N NaOH; aliquots of the homogenate were removed for radioactivity determination and determination of protein content according to the procedure of Lowry et al. (13).

Protocol II: In experiments using Protocol II, four matching pairs of cerebral hemispheres were prepared in close succession. Each chick forebrain was immersed in medium II at room temperature and divided along the longitudinal fissure with a thin razor blade. The composition of medium II is 155 mM NaCl, 5.6 mM KCl, 2.16 mM $CaCl_2$, 2.4 mM $NaHCO_3$ and 11.1 mM D-glucose; this composition is identical to that of the medium employed by Adey and Blackman and their colleagues for preparing chick cerebral hemispheres and bathing them during exposure to 147 MHz radiofrequency radiation. Each cerebral hemisphere was weighed and transferred into a polystyrene culture tube containing 1.0 ml medium II with 0.5 microCuries $^{45}Ca^{++}$ per ml. After 30 min. incubation in a 37°C. water bath, the radiolabelling medium was pipetted off and 2.0 ml medium II added to the tube. The tube was inverted into a 10 cm. square gauze pad, and the retained cerebral hemisphere quickly washed twice with 250 ml medium II. Each hemisphere was then transferred into a new polystyrene culture tube

containing 1.0 medium II. A set of four culture tubes, each with a ^{45}Ca-radiolabelled cerebral hemisphere, was placed in one styrofoam support, and another set of four tubes, each with a matching, radiolabelled cerebral hemisphere, was placed in a second styrofoam support. One support was placed inside the test chamber and the other support inside the control chamber.

After actual or sham exposure of the cerebral hemispheres in the test chamber for 20 min. coincident with co-incubation at 37°C. of the cerebral hemispheres in the control chamber, an 0.2 ml aliquot was removed from each tube for radioactivity determination. Upon removing the remainder of the medium, the cerebral hemisphere was homogenized with a Teflon/glass homogenizer in 2.0 ml 0.1 N NaOH and 0.1% (w/v) sodium dodecyl sulfate; an aliquot of the homogenate was removed for radioactivity determination.

Analytical Methods: All radiolabelled media and suspensions were mixed with Aquasol-2 (New England Nuclear, Boston, Ma.) and radioactivity determined (with automatic compensation for differential quenching) in a Beckman liquid scintillation counter. Protein concentrations were determined by the method of Lowry et al. (13) using bovine serum albumin as a standard.

Materials: Essential and non-essential amino acid mixtures were purchased from MA Bioproducts, Md.; L-glutamine, sodium pyruvate, sodium fumarate and bovine serum albumin from Sigma Chemical Co., St. Louis, Mo.; and $^{45}CaCl_2$ from New England Nuclear.

RESULTS

Protocol I was selected in the initial experiments to prepare chick brain tissue slices for exposure to radiofrequency radiation. The results of seven separate experiments using Protocol I are presented in Table 1. Ca^{++} efflux from the tissue slices is expressed as (1) the percentage of total $^{45}Ca^{++}$ initially present in the slices that was released under exposed and control conditions and (2) the amount of $^{45}Ca^{++}$ released per μg tissue protein under exposed and control conditions. All expressions of Ca^{++} efflux under either exposed or control conditions are averaged from four determinations in each experiment. When, for each experiment, the average percentage value representative of the exposed condition is divided by the average percentage value representative of the control condition, we find the average $\pm$ standard deviation value of this ratio from the seven experiments to be 0.99 ± 0.10. Analysis of these seven ratio values by a Student's t-test indicates that there is no significant difference between percentages of $^{45}Ca^{++}$ efflux under exposed and control conditions. When, for each of the latter four experiments, the average cpm ^{45}Ca released/ g tissue protein in the exposed condition is divided by the corresponding value representative of the control condition, we find the average $\pm$ standard deviation value of this ratio from the four experiments to be 0.94 ± 0.28. Analysis of these four ratio values by a Student's t-test indicates that there is also no significant difference between $^{45}Ca^{++}$ efflux under exposed and

control conditions when such efflux is expressed relative to tissue protein.

The results of five separate experiments in which Protocol I was used for the preparation and actual exposure of chick cerebral hemispheres are presented in Tables 2 and 3. Ca^{++} efflux from the cerebral hemispheres is expressed in Table 2 as the percentage of total $^{45}Ca^{++}$ initially present in the hemispheres that was released under exposed and control conditions, and in Table 3 as the amount of $^{45}Ca^{++}$ released/g tissue (wet weight) under exposed and control conditions. In each row of Tables 2 and 3, the form of expression of Ca^{++} efflux under exposed and control conditions pertains to a matching pair of hemispheres. When, for each row, the percentage value for the exposed condition is divided by that for the control condition, we find the average $\pm$ standard deviation value of this ratio from the 20 separate measurements to be 0.96 ± 0.19. Analysis of these 20 ratio values by a Student's t-test indicates that there is no significant difference between percentages of $^{45}Ca^{++}$ efflux under exposed and control conditions. When, for each row, the ^{45}Ca released/g tissue in the exposed condition is divided by the corresponding value representative of the control condition, we find the average $\pm$ standard deviation value of this ratio from the 20 separate measurements to be 0.92 ± 0.26. Analysis of these 20 ratio values by a Student's t-test indicates that there is also no significant difference between $^{45}Ca^{++}$ efflux under exposed and control conditions when such efflux is expressed relative to tissue mass.

Table 4 presents the results of two experiments in which Protocol II was used for the preparation and sham exposure of chick cerebral

hemispheres. When the data is analyzed in a manner identical to that employed for the data in Tables 2 and 3, we find the average $\pm$ standard deviation value of the percentage ratio from the 8 separate measurements to be 1.02 ± 0.14, and that of the cpm/g tissue ratio to be 1.08 ± 0.25. Application of the Student's t-test indicates that there is no significant difference between $^{45}Ca^{++}$ efflux under sham exposed and control conditions, independently of the manner in which such efflux is expressed.

DISCUSSION

The principal finding of this study is that there is no evidence to indicate that exposure of chick brain tissue to 147-MHz radiation, amplitude modulated at 16 Hz and applied at a power density of 0.75 mW/cm^2, significantly perturbs $^{45}Ca^{++}$ efflux. The significance of this negative evidence is that the data was secured from two distinct protocols for preparing and radiolabelling the chick brain tissue. It is, therefore, important to consider the strengths and weaknesses inherent in the experimental design of each protocol with respect to testing the effect of radiofrequency radiation on Ca^{++} efflux.

The primary advantage of the experimental design of Protocol I is that the brain tissue is rapidly reduced, following euthanasia of the animal, to dimensions optimal for short-term in vitro support. Tissue slices with dimensions of 1-2 mm are of a size that permits passive diffusion of dissolved gases, nutrients and metabolic waste products into and out from the slices during the time periods required to radiolabel the cells and subject them to radiofrequency radiation. The secondary strengths of Protocol I are that (a) the tissue slices are incubated in a medium whose nutrient and dissolved gas composition can support the characteristic, high metabolic rate of nervous tissue cells and (b) all the tissue slices in each experiment are simultaneously radiolabelled with $^{45}Ca^{++}$ in the same culture tube. The major disadvantage of Protocol I is that multiple determinations of $^{45}Ca^{++}$ efflux from control and exposed slices must be made in each

experiment and an averaged expression of $^{45}Ca^{++}$ efflux calculated from these determinations.

By contrast, the primary advantage of the experimental design of Protocol II is that anatomically matched pairs of brain tissue are prepared, and consequently $^{45}Ca^{++}$ efflux from one member of a pair can be directly compared to such efflux from the other member. A secondary strength of Protocol II is that the gross architecture of the brain tissue is not markedly destroyed during preparation. However, the major disadvantages of Protocol II are that (a) chick cerebral hemispheres are too large to permit adequate, passive diffusion of dissolved substances to and from the central region of the hemispheres and (b) the radiolabelling of paired hemispheres will be radiolabelled to slightly different extents (normal experimental variability would suggest a difference of 5-10% on a per weight basis). Although this latter disadvantage may appear initially inconsequential, it serves, in essence, to define the minimal difference that one can experimentally determine between the paired hemispheres. Consequently, a consistent, small percentage difference in the radiolabelling of paired hemispheres could either mask a genuine, equal but opposite, effect of radiofrequency radiation on Ca^{++} efflux or artefactually manifest the appearance of such an effect.

In general, therefore, the major advantages in the experimental design of each protocol address the major disadvantages in the design of the other protocol. Furthermore, the procedures conducted for the preparation and radiolabelling of cerebral hemispheres in Protocol II reproduce as closely as possible those conducted by Adey and Blackman

and their colleagues in their studies. Nonetheless, neither protocol yields data consistent with the argument that appropriately modulated 147 MHz radiation perturbs Ca^{++} efflux from chick brain tumors. This negative finding applies when $^{45}Ca^{++}$ efflux is expressed on a tissue mass basis, as Adey and Blackman have done, or on a percentage basis.

It is also of interest to note that application of the Student's t-test to the raw data in Table 2 (that is, analysis of the difference between the cpm released/g tissue data for the exposed condition with that for the control condition) shows that there is no significant difference between the two columns of data, which is the same conclusion drawn when similar analysis is applied to the 20 test/control ratio values. However, application of the Student's t-test to the inverted ratio values (that is, the control/test ratio values) yields a t-statistic of 2.37, a magnitude that suggests there is a significant difference at the 98% confidence level between the $^{45}Ca^{++}$ efflux under control and test conditions. It has been suggested that it is inappropriate to subject the ratio values calculated from this kind of study to a parametric statistical analysis involving a t or F distribution; the specifics of our raw data suggest that such analysis may indeed lead to ambiguous conclusions (14).

Blackman and his colleagues, in particular, have conducted very extensive studies demonstrating enhanced Ca^{++} efflux during exposure to radiofrequency radiation with only highly specified carrier and amplitude modulation frequencies and power density ranges. In view of their reports, we believe that the most prudent interpretation of our findings is that the exposure conditions we have used are not

sufficient by themselves to enhance Ca^{++} efflux. In other words, it is possible that Blackman and his colleagues report positive findings and we negative findings, using identical electromagnetic fields, because Blackman's group introduces specific factors (such as the spacing and number of culture tubes in the test chamber) in their experiments which complement the radiofrequency radiation sufficiently to enhance Ca^{++} efflux. However, this possibility raises the issue that appropriate electromagnetic fields may or may not affect Ca^{++} fluxes in brain tissue depending upon the simultaneous presence of other physical factors separate from that of the non-ionizing radiation.

In conclusion, the data presented here demonstrates that the specific radiofrequency radiation used does not perturb Ca^{++} efflux from avian brain tissue under in vitro conditions. Comparison of this data with that gathered by Blackman's group, however, suggests that appropriate radiofrequency radiation in conjunction with certain other physical factors may indeed perturb Ca^{++} efflux.

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LEGENDS

Table 1: Results of experiments in which chick brain tissue slices were prepared by Protocol I and ^{45}Ca efflux determined upon incubation in the control and test chambers, with the tissue slices in the test chamber being exposed to 147-MHz radiation.

Tables 2 and 3: Results of experiments in which chick cerebral hemispheres were prepared by Protocol II and ^{45}Ca efflux determined upon incubation in the control and test chambers, with the cerebral hemispheres in the test chamber being exposed to 147-MHz radiation.

Table 4: Results of experiments in which chick cerebral hemispheres were prepared by Protocol II and ^{45}Ca efflux determined upon incubation in the control and test chambers, with the cerebral hemispheres in the test chamber being subject to sham exposure.

Table 1

Experiment	Percentage ^{45}Ca Released from Tissue Slices in Test Chamber	Percentage ^{45}Ca Released from Tissue Slices in Control Chamber	Test/ Control Ratio	cpm ^{45}Ca Released/µg Tissue Protein in Test Chamber	cpm ^{45}Ca Released/µg Tissue Protein in Control Chamber	Test/ Control Ratio
1	24.8 ± 3.1	23.0 ± 3.0	1.08			
2	15.4 ± 2.5	17.4 ± 4.7	0.89			
3	34.6 ± 2.1	32.2 ± 4.9	1.07			
4	45.6 ± 3.8	40.1 ± 0.7	1.14	0.97 ± 0.15	0.76 ± 0.29	1.28
5	38.3 ± 5.2	40.7 ± 8.7	0.94	0.80 ± 0.20	0.96 ± 0.36	0.83
6	26.4 ± 3.3	28.3 ± 4.8	0.93	0.82 ± 0.46	1.33 ± 0.94	0.62
7	24.1 ± 2.9	27.5 ± 2.1	0.88	0.67 ± 0.20	0.65 ± 0.08	1.03

Table 2

Experiment	Percentage ^{45}Ca Released from Cerebral Hemisphere in Test Chamber	Percentage ^{45}Ca Released from Cerebral Hemisphere in Control Chamber	Test/ Control Ratio
1	19.7	18.4	1.07
	23.8	24.2	0.98
	21.2	20.8	1.02
	18.6	20.2	0.92
2	18.0	24.6	0.73
	19.0	19.4	0.98
	18.6	23.0	0.81
	21.5	22.9	0.94
3	17.7	25.3	0.70
	22.4	22.0	1.02
	18.8	23.9	0.79
	19.4	16.5	1.18
4	24.6	16.8	1.46
	17.5	22.8	0.77
	18.2	20.2	0.90
	25.4	21.6	1.18
5	15.5	20.9	0.74
	17.5	17.2	1.02
	23.3	19.6	1.19
	23.6	28.6	0.83

Table 3

Experiment	cpm ^{45}Ca Released/gm Tissue in Test Chamber	cpm ^{45}Ca Released/gm Tissue in Control Chamber	Test/ Control Ratio
1	5827	6592	0.88
	7541	9958	0.76
	6854	7609	0.90
	7295	7109	1.03
2	5481	8563	0.64
	6217	5804	1.07
	4935	8217	0.60
	4792	7120	0.67
3	6192	11500	0.54
	7760	10522	0.74
	6981	8615	0.81
	5288	4423	1.20
4	8517	7400	1.15
	8940	8962	1.00
	4769	7120	0.67
	10017	8034	1.25
5	5774	8200	0.70
	7271	5442	1.34
	8117	5917	1.37
	7065	6929	1.02

Table 4

Experiment	Percentage ^{45}Ca Released from Cerebral Hemisphere in Sham Chamber	Percentage ^{45}Ca Released from Cerebral Hemisphere in Control Chamber	Sham/ Control Ratio	cpm ^{45}Ca Released/gm Tissue in Sham Chamber	cpm ^{45}Ca Released/gm Tissue in Control Chamber	Sham/ Control Ratio
1	16.4	19.5	0.84	6204	8364	0.74
	19.3	18.3	1.05	5583	6217	0.90
	16.5	15.9	1.04	7260	6129	1.18
	22.9	18.2	1.26	7074	5058	1.40
2	17.9	16.3	1.10	5162	5299	0.97
	15.6	17.7	0.88	3966	4369	0.91
	16.9	17.9	0.94	5172	4783	1.08
	16.8	16.7	1.01	6730	4560	1.48

MORPHOLOGICAL CHANGES IN CEREBELLUM OF NEONATAL RATS EXPOSED TO 2.45 GHz MICROWAVES

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Abstract

One-day and six-day old Sprague-Dawley rates were exposed in the far field to 2.45 GHz (cw) microwaves at 10 mW/cm^2 for five consecutive days, 7 hours per day (SAR 2W/kg). Pups were euthenized one day after exposure and the cerebella processed for light and electron microscopy. Matching cerebellar sections and folia from irradiated and sham irradiated animals were examined. Light microscopic examination revealed the presence of small deeply-stained cells with hyperchromatic pyknotic nuclei within the external granular layer (EGL). The number of these pyknotic cells in the experimental animals was nearly twice that in the controls. The Nissl bodies in Purkinje cells were finely dispersed. In some experimental animals mononuclear cellular infiltration was demonstrated. Under the electron microscope the deeply-stained pyknotic small cells presented electron dense nuclei with clumped chromatin, extrusion or disintegration of the nucleus, ruptured nuclear membrane the vacuolization of the cytoplasm. Eventually these cells became phagocytosed by surrounding EGL cells. Most of the Purkinje cells of experimental animals showed small, disorderly arrays of rough endoplasmic reticulum (RER) instead of the typical orderly stacks of parallel arrays. These observations suggest that microwave radiation may interfere with early genesis of cerebellar microneurons and alter the metabolic status of Purkinje cells. However, this effect might be reversible.

Introduction

The effects of high power density non-ionizing radiation on adult mammalian brain have been reported by many authors (Oldendorf, 1949; Minecki and Bilski, 1961; Cholodov, 1966; Tolgskaja and Gordon, 1971). These effects consisted of degenerative lesions in nerve cells and white matter, hyperemia, inflammatory and glial reactions in the brains of rabbits, rats and mice exposed to high power density microwave irradiation and high intensity magnetostatic fields. Exposure of Chinese hamsters to 2.45 and 1.7 GHz microwaves at low and intermediate power densities (10, 25, and 50 mW/cm^2) resulted in vacuolization, chromatolysils, scarcity of rough endoplasmic reticulum (RER) and polyribosomes in neuronal somas in hypothalamic and subthalamic nuclei (Albert and Desantis, 1975).

Gross effects of non-ionizing radiofrequency electromagnetic (RFEM) radiation on fetal development have also been reported. These include occurrence of hemorrhage, resorption, exencephaly and fetal death in conceptuses of CF-1 mice exposed, during gestation, to 2.45 GHz microwaves at 123 mW/cm^2 (Rugh et al., 1974; Rugh et al., 1975; Rugh and McManaway, 1976). Shore et al. (1977) reported no effect on the litter size, but did note a decrease in body and brain mass in rats exposed to 2.45 GHz microwaves at 10 mW/cm^2 for five days during gestation. Berman et al. (1978) exposed CD-1 mice to 2.45 GHz radiation for 100 minutes daily at power densities that ranged from 3.4 to 28 mW/cm^2. They observed a decrease in mean body mass and an increased incidence of exencephaly among fetuses exposed at the highest (28 mW/cm^2) power density. Thus, the existing studies on the effects of non-ionizing radiation on developing fetus have examined gross teratology, body mass, litter size and uterine resorptions, but none have addressed the question of cellular an subcellular changes.

Recently, this laboratory explored the effects of 2.45 GHz (10mW/cm^2) and 100 GHz (46 mW/cm^2) non-ionizing radiation on the developing rat cerebellum. There was no effect on the size of the cerebellum. The proportion between the cerebellar cortical layers and the white core was the same in both experimental and control animals but there was a statistically significant decrease in the relative number of Purkinje cells of rats irradiated in

utero, or during early postnatal life (Albert et al. 1981a). However, the decrease in Purkinje cell population was insignificant when the animals were irradiated during early postnatal life and left for 40 days to recover. These findings raised questions about the mechanisms of radiation effect in rats resulting in numerical reduction of Purkinje cells. A similar study on Monkey cerebellum showed absence of any effects on Purkinje cells (Albert et al 1981b).

It has been reported that agents interfering with normal development of cerebellar granule cells from EGL will subsequently affect the cerebellar neurones such as Purkinje cells as a result of loss of granule cells (Altman, 1972; Altman and Anderson, 1972; Jacobson, 1978). Therefore, the present study was designed to investigate the possible effects of low level non-ionizing RFEM radiation on the EGL of the neonatal rat cerebellum with special emphasis on Purkinje cells.

Materials and Methods:

Twenty-four newly born Sprague-Dawley rats were used in this study; eight pups were one-day old, sixteen were six-days old. Two separate anechoic chambers were utilized to provide exposure to RFEM radiation or sham (control) exposure. During exposure, animals were housed in plexiglas containers with internal dimensions of 19 x 11.4 x 7.6 cm. The tops and bottoms of these containers consisted of removable polystyrene grids to permit proper ventilation. Each container was positioned reproducibly on a plexiglas rack 208 cm. below a trunkated horn antenna. Sham-exposed animals were similarly housed in a separate anechoic chamber.

In an effort to define the exposure of individual subjects, a series of three field intensity measurements were made within each of the plexiglas containers. The measurements were made with and without nonexperimental subjects in the remaining containers. All measurements were made with nonperturbing, three dimensional probe (Collins S/N 57) and EIT digital receiver (S/N 1004).

It was determined that exposures varied as function of position within the container and also with time due to field perturbations produced by the movements of other animals. Consequently, although the average field

intensity was 10 mW/cm^2, an animal might have received as little as 4 mW/cm^2 or as high as 30 mW/cm^2 at any instant. Utilizing the Radio-Frequency Radiation Dosimetry Handbook (Durney et al., 1977), specific absorption rate (SAR) for these rats was computed to be (2 mW/g).

Matching sets of litter mates from both groups of animals, the one-day and six day old pups, were placed in well ventilated plexiglas cages in anechoic chambers (as described above) for 3.5 hours (9:00 a.m. - 12:30 p.m.). One chamber was energized so that the pups were irradiated with 2.45 GHz (cw) microwaves at 10 mW/cm^2, while the other chamber was used for sham irradiation. After 3.5 hours of irradiation, the pups were reunited with their mothers outside the anechoic chambers for 1.5 hours for feeding. At 2:00 p.m., control and experimental pups were again placed in their respective chambers and irradiated for an additional 3.5 hours (2:00 p.m. - 5:30 p.m.). The pups were then returned to the cages with their mothers until the next morning. This procedure was repeated daily for five days. Thus the young rats were irradiated for 7 hours/day for five consecutive days. On the sixth day all animals were anesthetized by pentobarbital sodium (50 mg/kg) and fixed by intracardiac perfusion. Animals used for light microscopy were perfused with 10% buffered formalin. Those for electron microscopy were perfused with weak Karnovsky's fixative containing 1% paraformaldehyde, 1.25% glutaraldehyde, 0.12% $CaCl_2$ in Cacodylate buffer. After perfusion, the brains were dissected out, the cerebella were separated and left in the fixatives for 12 hours.

For light microscopy, the cerebella were bisected in the median plane, dehydrated, double imbedded in paraffin-celloidin and serially sectioned at 10 μm in the sagittal plane. Sections were stained alternatively with hematoxylin and eosin and 1% thionin in Wolpole acette buffer at pH 4.8 for Nissl substance. The entire series of sections from eperimental and conrol animals were matched and examined according to predetermined 5 parasagittal planes (Albert et al., 1981). This step was necessary to insure that identical areas of the cerebellar folia and EGL were examined in both experimental and control animals.

For electron microscopy, thin sagittal slices (1-2 mm thick) were cut from the cerebella at the vermal and

paravermal regions. Small wedges from the cortical area of matching folia were cleanly dissected and processed for electron microscopic examination.

Observations and Results:

I. Light microscopic examination of cerebella of irradiated (experimental) and sham irradiated (control) animals revealed the following:

a) Along with mitotic cells, there were many small deeply stained cells scattered among the developing normal microneurone in the EGL of both experimental and control animals. However, there was a consistently greater preponderance of these cells in the experimental animals than in controls. The deeply stained cells possessed hyperchromatic pyknotic nuclei, usually rounded and centrally placed. In some cells the nuclei were eccentric, indented or fragmented (Fig. 1). These observations are indicative of various stages of degeneration. The degenerating cells were scattered throughout the EGL. These cells were counted in 3 serial parasagittal sections at 5 different matching planes of the cerebella of both experimental and control animals. The results indicated a statistically significant increase in the relative number of pyknotic cells per folium in the irradiated animals. Their number in the experimental animals was approximately twice as much as their number in the control animals (Table).

b) In addition to the changes in the EGL cells, there was a change in the pattern of Nissl bodies of many Purkinje cells in experimental animals in comparison to controls. The Nissl bodies were in the form of finely dispersed granules instead of the compact rod shaped appearance generally seen under the light microscope in normal Purkinje cells (Fig. 2). However, tyical chromatolytic patterns were not observed.

c) Some folia of the irradiated animals were studded with mononucleated blood cells, usuallly near a capillary blood vessel in the white core. The mononucleated cells were dispersed over the internal granular layer as well. Neither extravasation of red blood cells, nor any pathological changes of the blood vessels were observed. It is noteworthy to mention that the vascular cellular infiltration was not unique to that of any specific or non-specific inflamatory conditions.

II. Electron microscopic observations:

a) The pyknotic cells observed under light microscopy presented large rounded nuclei compactly packed with homogenous electron dense material (heterochromatin), concealing any nuclear details. Some of these cells presented ruptured nuclear membrane with leakage of the nuclear contents in a dense cytoplasm. Some cells showed extrusion of the nucleus with polarized compact heterochromatin and normal appearance of euchromatin. Others presented disintegrated nucleus with vacuolated cytoplasm (Fig. 3). This is interpreted as various stages of degeneration among the neuroblasts in EGL of irradiated animals. It was not uncommon to find the degenerated cells engulfed by other surrounding EGL cells, probably glioblasts.

b) On the other hand, most of the Purkinje cells of exposed animals mainly showed small, disorderly arrays of rough endoplasmic reticulum (RER) instead of the typical orderly stacks of parallel arrays (Fig. 4a,b). Nevertheless, such observation among Purkinje cells of irradiated animals is not suggestive of definite chromatolysis.

Discussion:

In this study, exposure of neonatal rats to 2.45 GHz (cw), 10 mW/cm^2 microwave irradiation resulted in some degenerative changes among EGL cells. The appearance of these degenerating cells is somewhat similar to those reported in rat EGL after postnatal exposure to x-ray irradiation (Altman et al., 1969; Altman and Anderson, 1972; Das, 1977). However, the incidence of such degenerating cells after exposure to non-ionizing microwave radiation is far less than that obained by x-irradiation. On the other hand, degenerating cells were reported in EGL of the control animals as well. However, their number in the experimental animals was nearly double that in the controls. It is now generally accepted that histogenic cell death normally occurs during development of the nervous system (Jacobson, 1970 and Cowan, 1973). It is strongly contingent on conditions outside the cells that die; e.g., interaction with other cells, nutritional, hormonal and trophic influences. It seems therefore that microwave irradiation might have induced a destructive

effect on the developing EGL cells which resultd in increased histogenic cell death in irradiated animals. However, the relative number of degenerated cells per cerebellar folium of the experimental animals is not sufficient to produce teratogenic manifestations. This view may provide a substratum to explain a variety of results ranging from decrease in body and brain mass, without any anomalies, at low power densities up to exencephaly, resorption and fetal death at higher power densities (Rugh et al., 1975; Rugh and McManaway, 1976; Berman et al., 1978). The different irradiation parameters could be the cause of these variations but the nature of the degenerative effect of radiation is still present in all the experiments conducted. Albert et al. (1981) reported a decrease in Purkinje cell population when the animals were exposed to 2.45 GHz (cw) at 46 mW/cm^2 microwave radiation in utero and during early potnatal period. The possibility that microwave radiation might have interfered with the early genesis of developing neurons may be the underlying cause behind this finding.

Examination of the cytology of Purkinje cells in the exposed cerebella revealed a change in the pattern of their Nissl substance. At the light microscopy level the tigroid substance appeared finely dispersed in the cytoplasm as if it had been disintegrated. This was confirmed at the electron microscopy level; the RER appeared in the form of a few small disordered arrays. On the contrary, the RER of Purkinje cells of control animals was in the form of orderly stacks of parallel arrays characteristic of normal Purkinje cells (Palay and Chan-Palay, 1974). This finding is in accord with Hansson (1981) who reported alteration of RER and disintegration of Nissl bodies of Purkinje cells in rabbits exposed to E field of 14 kV/m (undisturbed field, 50 Hz AC). The change in the pattern of Nissl substance and RER is indicative of alteration in the metabolic status of the cell, especially the common protein metabolic pool. Although this could be an early sign of degeneration, there was no evidence of definite chromatolytic changes. This is in favor of a reversible effect of non iodizing (microwave) radiation at low power density. This finding may provide an explanation for the results previously obtained in our laboratory (Albert et al., 1981). There was a statistically significant decrease in Purkinje cell population when the animals were sacrificed immediately after exposure to microwave radiation with specifications similar to the experiment conducted in this study.

However, the decrease in Purkinje cell number was insignificant when the animals were allowed to recover for 40 days after cessation of irradiation.

In other experiments, pykosis, chromatolysis, vacuolation of cytoplasm and paucity of free ribosomes and RER in hippocampal, thalamic and hypothalamic neurones in adult Chinese hamsters exposed to electromagnetic radiation at power densities 10 and 25 - 50 mW/cm^2 (Albert and de Santis, 1975 and McKee et al., 1980). However, these authors did not mention any change in cerebellar Purkinje cells. The morphological change reported in these studies and the findings of the present study are more or less unique in spite of the variation of the site of change itself. This variation could be attributed to the difference in vulnerability of the various neuronal systems of different species during different ages to EM radiation.

During examination of the material in this study some folia of the irradiated animals showed extravasation of some mononucleated blood cells in the vicinity of a capillary vessels in the white core. No change in the vascular walls was observed. This finding argues with the observations reported from the studies on the effect of electro-magnetic radiation on the blood-brain barrier (BBB) (Oldendorf, 1970; Frey et al., 1975; Oscar and Hawkins, 1977; Albert, 1977 and 1978). These authors suggested an increase in the permeability of blood brain barrier (BBB) due to irradiation. However, no opening in the tight junction between the endothelial cells of the brain vasculature was reported in spite of the increased passage of injected horse-radish peroxidase tracer through BBB of irradiated animals (Albert and Kerns, 1981).

It is evident that there is an obvious need for additional research on the mechanisms of interaction of electromagnetic radiation with the nervous system although the effect of exposure to low intensity radiation seems to be reversible.

Fig. 1 Photomicrographs a & b of parasagittal sections in rat cerebellar cortex showing deeply stained degenerated cells with hyperchromatic, pyknotic nuclei within the external granular layer.

Fig. 2 Photomicrograph of cerebellar Purkinje cells stained with thionin. a) Purkinje cells from control animal showing the compact packing of rod shaped Nissl substance. b) In constrast Purkinje cells from irradiated animal show finely dispersed Nissl bodies.

Fig. 3 Electron micrographs of the deeply stained cells within EGL of experimental animals. a) Nuclei showing clumped chromatin. b) Extruding nucleus. c) A cell with disintegrated nucleus and vaculated cytoplasm (arrow). d) Phagocytosed degenerated cell. (Note the ruptured nuclear membrane.

Fig. 4a Electronmicrograph of a Purkinje cell from a control animal. Arrows outline orderly stacks of RER.

Fig. 4b Electronmicrograph of a Purkinje cell from an irradiated animal. Arrows point to the disorderly arrays of RER of the experimental animal. (Note the lack of the typical stacks of RER).

Effect of 2450 MHz (CW) microwaves at 10 mW/cm^2 on the external granular layer (EGL) of rat cerebella irradiated during day 1 - 5 and 6 - 10 postnatally*.

		Total No. of degenerated cells/ parasagittal section.	Relative No. of degenerated cells/folia in parasagittal section.	Difference between relative No. of degenerated cells in experimental and control cerebella.	Proportion of increase of relative No. of degenerated cells in experimental animals.
Animals irradiated day 1-5.	Experimental	52.1 SD±24.78	5.17 SD±1.89	2.22 SED±1.62	1:1.75
	Control	27.78 SD±10.5	2.95 SD±0.81	0.001<P<0.01	
Animals irradiated day 6-10.	Experimental	128.30 SD±35.01	11.76 SD±3.83	5.23 SED±3.11	1:1.80
	Control	76.16 SD±19.7	6.53 SD±1.71	0.02<p<0.05	

* All values are the mean number of degenerated cells counted in parasagittal sections at different planes studied in each group.

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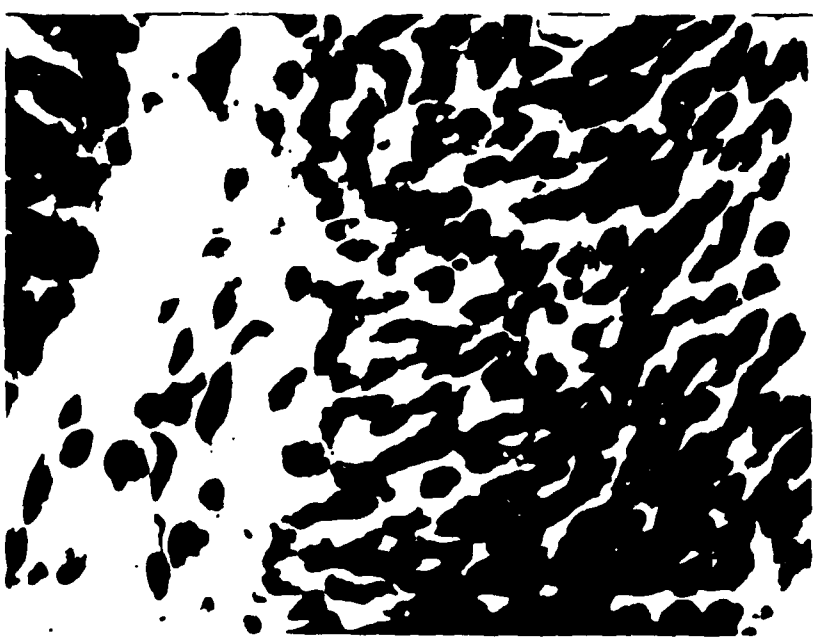
1a



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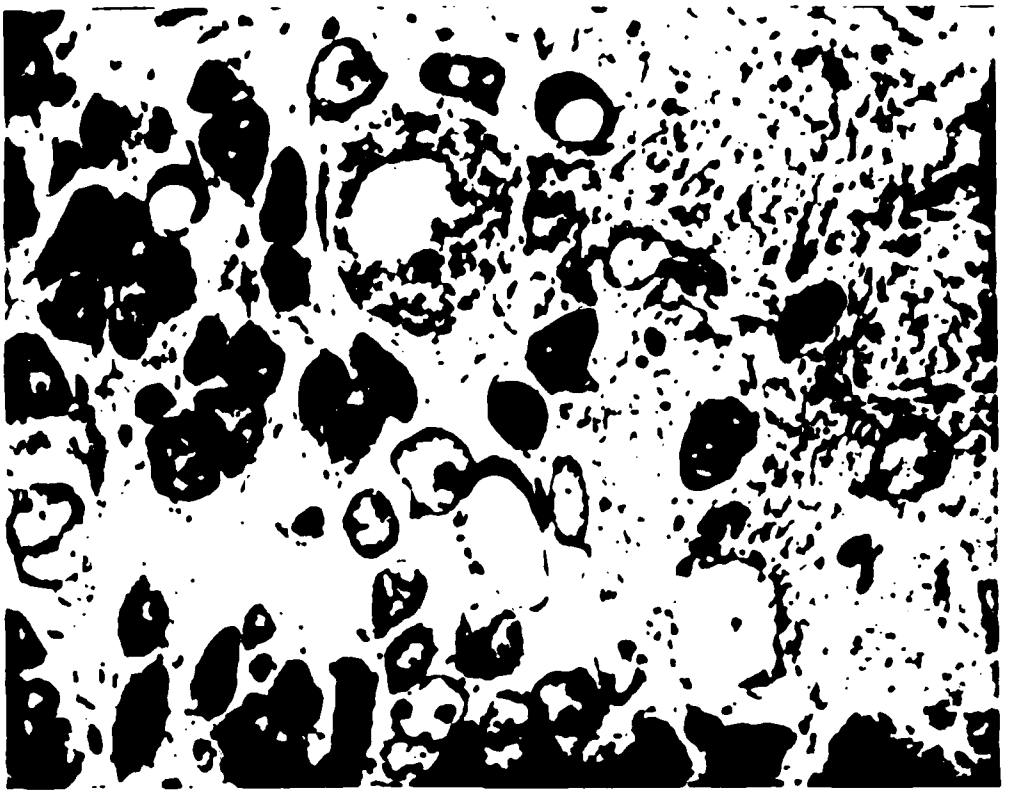

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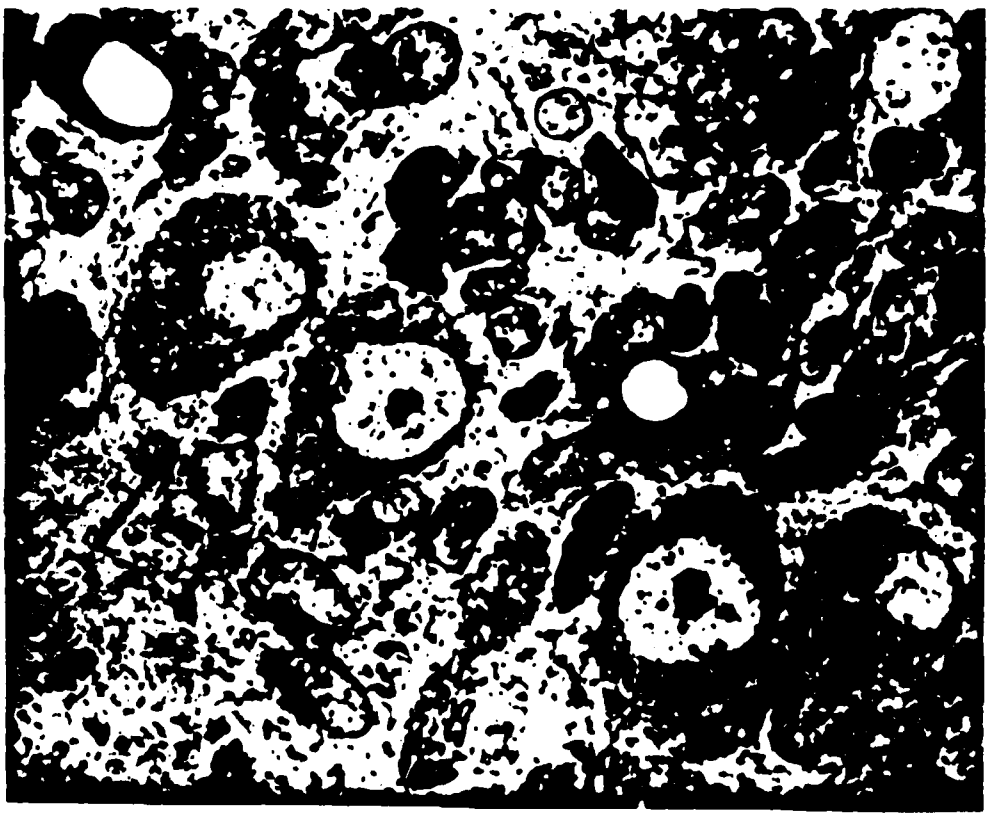

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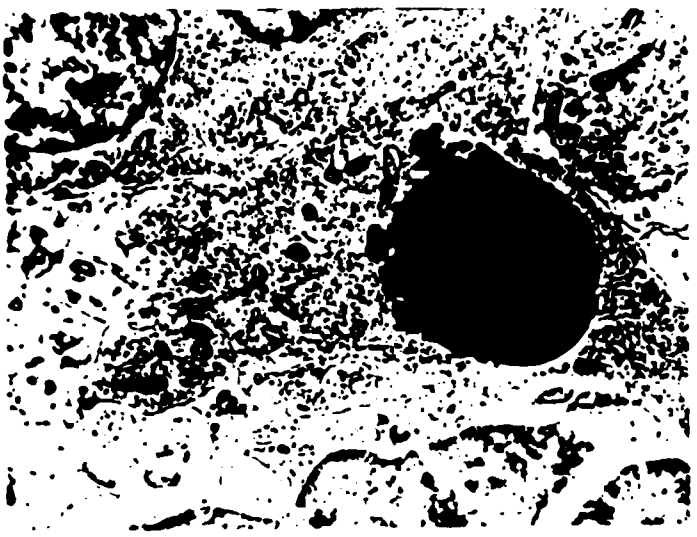

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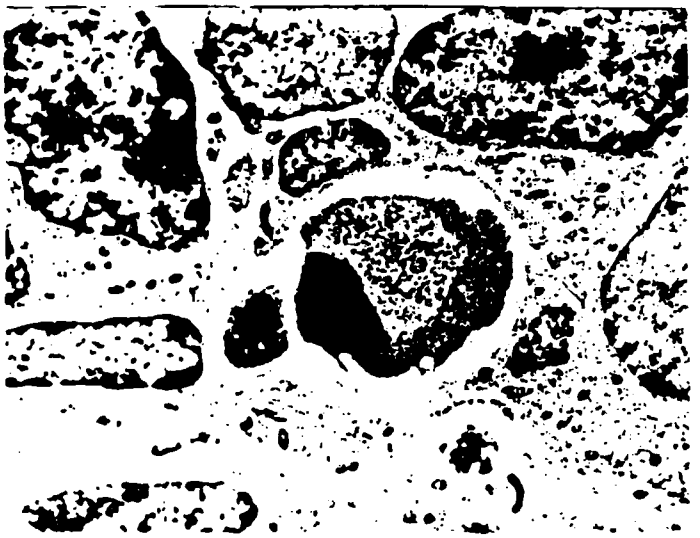

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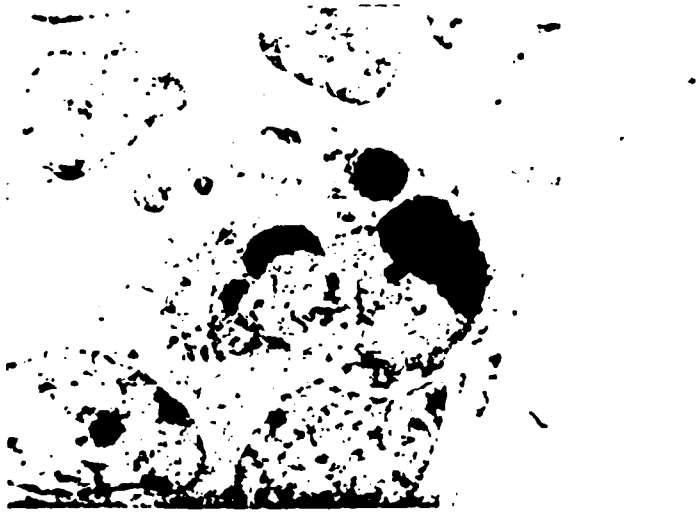

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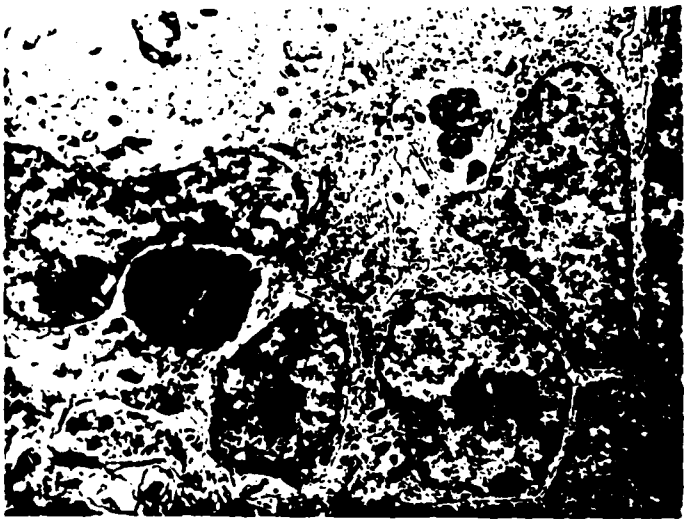

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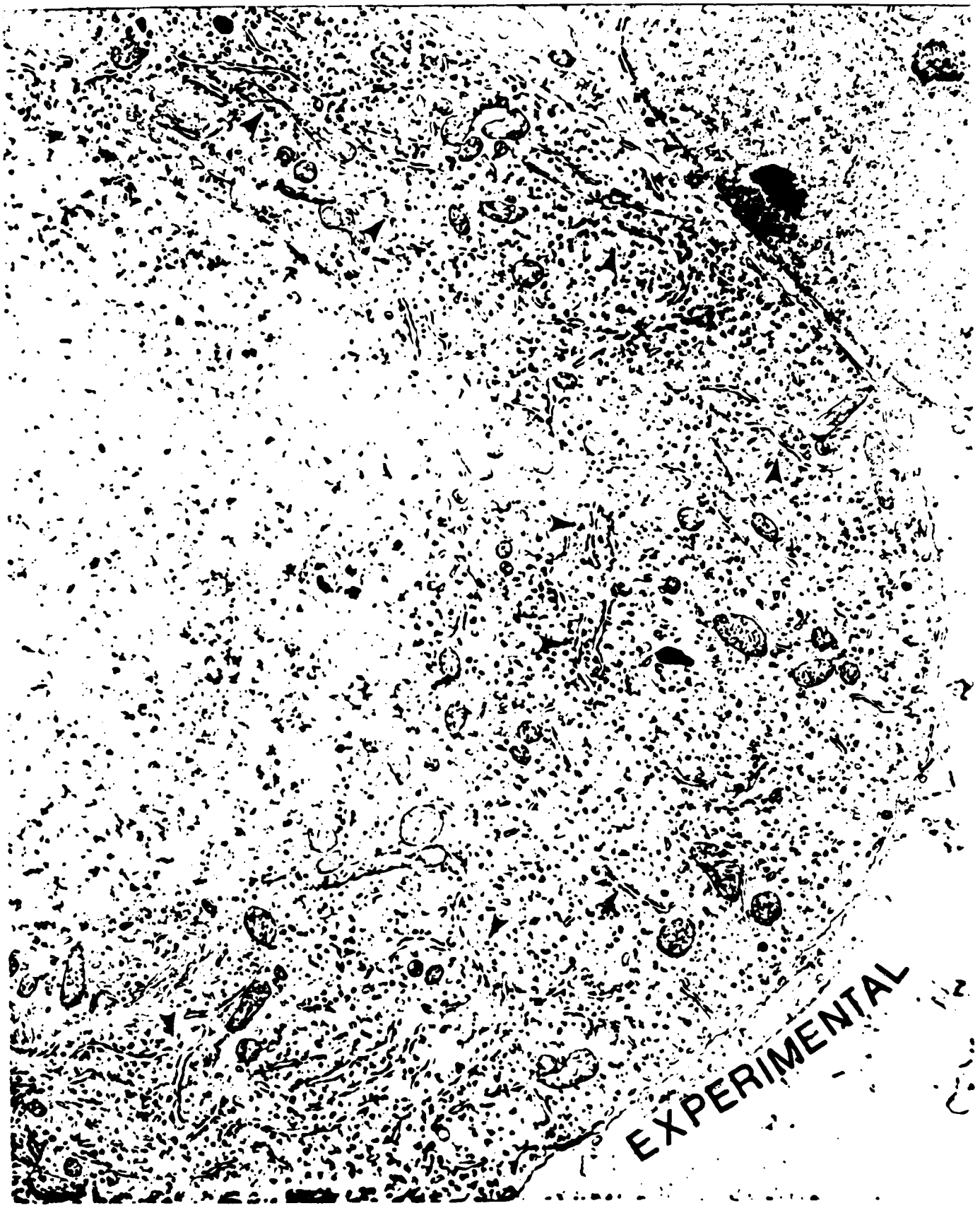
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