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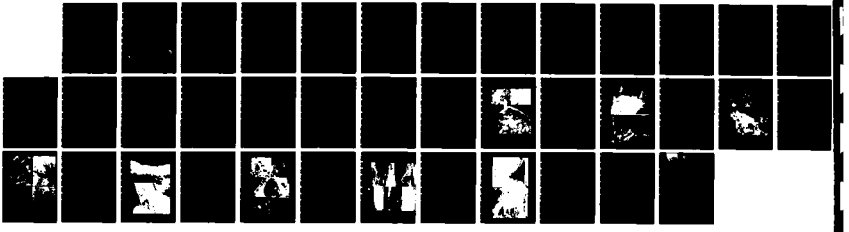

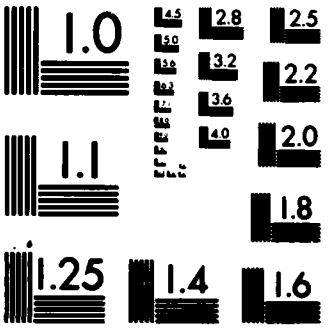

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MICROWAVE AND TEMPERATURE EFFECTS ON THE MURINE OCULAR LENS IN VITRO:

Annual Summary Report

DR. JOHN R. TREVITHICK

June, 1981

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND

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

Rat ocular lenses were studied after fixation and critical point drying of the tissue by scanning electron microscopy (SEM) following exposures to elevated temperatures and/or microwave irradiation in a thermostatically controlled chamber. In this way, the temperature of the lens bathing medium was set independently of the temperature increase normally associated with application of microwave power. Irradiations were done at three final temperatures and three specific absorption rates (SAR) for two durations. These were accomplished at 915 MHz in WR975 waveguide with either pulsed (Pu) or continuous wave (CW)

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radiation of equal average power. The parameters of the (Pu) radiation were selected to maximize the production of thermoacoustic expansion.

In addition to the types of damage noted on previous progress reports: (1) immediately after pulsed microwave irradiation, holes in cell membranes (2) after incubation for 48 hr, globular degeneration including very large globules at high SAR's and 37°C, this work has investigated possible reciprocity between time and SAR: - although dose rate and time were varied from 5 to 20 minutes, for the same total dose a similar degree of globular degeneration (a measure of cataractous damage) was observed. More conditions and time need to be investigated to confirm this observation.

Summary

Over the three year period of this grant, the initial aims of this project were to develop techniques for incubating intact rat lenses in vitro in order to study the development of cataracts when lenses are exposed to elevated temperatures and/or microwave irradiation. We planned to (1) establish cataractogenic temperatures and SARs for irradiation in vitro and (2) investigate the mechanisms of cataractogenesis in such lenses.

Initial studies indicated a linear correlation between depth of cataractous globular degeneration and temperature when the lens was exposed to a short period of temperature elevation and postincubated for a period of 48 hr. This unexpected linear relationship was found between 37° C and 50° C; in addition, at 47° C and 50° C some very large globules were formed. Surprisingly, at a higher temperature (60° C) the lenses had normal opacity and acuity, apparently because they had been fixed by the high temperature. D-tocopherol acetate when added to lenses before incubation at 41° C, prevented most of the globular degeneration observed at this temperature.

In initial attempts to expose the lenses to microwaves, a system was devised to rapidly circulate thermostatted coolant around the lens while it was being irradiated. This system permitted experimental separation of heating effects in the lens from effects due to electromagnetic radiation, since there was no measurable temperature elevation in the lens with respect to the surrounding medium even at highest microwave exposure levels tested. Irradiation was performed for two exposure times and at three SAR values:

The results of the irradiation indicated that the effect of the electromagnetic radiation itself could be considered to be equivalent to heating, since at the highest dose rate and 37° C, large globules were formed, which would otherwise have been reported only at 47°, equivalent to a temperature elevation of 10° C. Pulsed irradiation at high SAR values appeared to result in holes in the surface of cells, a novel observation which could indicate that thermoacoustic expansion is causing mechanical damage to cell membranes. A preliminary estimate of amount of damage experienced as a result of total dose level of microwaves is consistent with the idea that the amount of damage is roughly proportional to the total dose delivered to the lens, and that a reciprocal relationship exists between dose rate and time required to cause a defined amount of globular degeneration. More work needs to be done to explore the possibility of such reciprocoty, which has important implications for personnel who are chronically exposed to low levels of microwaves as well as those suffering from the effects of acute exposure.

Foreword

A. List of Professional Personnel Employed on This Project

Principal Investigator	-	Dr. John R. Trevithick, Ph.D.
Research Associate	-	Dr. P. Jill Stewart-DeHaan, Ph.D.
Research Associate	-	Dr. William M. Ross, Ph.D. (left Dec. '80)
Research Associate	-	Dr. Madhu Sanwal, Ph.D. (began Jan. 81)

B. Animal Care

In conducting the research described in this report, the investigator adhered to the "Guide for Laboratory Animal Facilities and Care" as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Science - National Research Council, U.S.A.

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The reader is referred to previous progress reports for figures showing work cited in "previous results".

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Introduction

Since the collaborative development of an *in vitro* technique for experimentally distinguishing the cataractogenic effects of microwaves due the (1) elevated temperatures and (2) the electromagnetic field (Stewart-DeHaan et al, 1980) several generally accepted concepts regarding microwave cataractogenesis have been felt to deserve more detailed scrutiny. Such ideas as a temperature threshold for microwave cataractogenesis, which have been developed almost exclusively for CW microwaves, have ignored the possibilities for mechanical damage to the eye and its lens as a result of the thermoacoustic expansion caused by high energy pulsed microwaves (Pu) similar to those commonly in use in military radars.

In the experiments reported here, we used the system we have devised for microwave irradiation of rat lenses *in vitro*. This permits the temperature of the lens bathing medium to be varied independently of the irradiating field, and examination of the lens by SEM following fixation (1) immediately after irradiation, or (2) following 48 hr incubation in M199. The changes are compared with known changes involved in the course of cataractogenesis induced by several toxic agents such as elevated glucose levels in M199, elevated temperature alone, or toxic drugs such as hygromycin B.

In these experiments we wished to approach in a preliminary way the question: "Is there a reciprocal relationship between time and dose rate of microwaves (defined as Specific Absorption Rate, S.A.R.) required to induce a certain amount of cataractogenic damage in a lens."

In addition we present preliminary data outlining (1) several biochemical analytical techniques we have been developing to permit analysis of ATP, NADPH, NADH, vitamin E, O_2^- superoxide radical levels and lens protein alterations during the course of the cataractogenesis and (2) high voltage electron microscopy which we hope to apply to single lens fiber cells from the equatorial area in which the initial changes in microwave cataracts occur.

Materials and Methods

a) Microwave Exposure Apparatus

Rats (150-200 g) were sacrificed by decapitation and the lenses removed. Lenses with intact capsules were placed in M199 in culture tubes or in an apparatus in which they could be bathed in circulating bicarbonate or phosphate buffered saline (PBS) at controlled temperatures while simultaneously being subjected to microwave radiation. Intact lenses were exposed to elevated temperatures for periods of up to one hour, followed by incubation in M199 at 35.5°C for two days. Some lenses were pre- and post-exposure incubated in M199 with 2.4 μM α-tocopherol added in order to explore possible prophylatic effects for exposure to elevated temperatures.

Microwave radiation was delivered to the lens holder in two modes, Pu and CW. The lens holder is shown in the waveguide structure in Fig. 1 and the exposure system block diagram in Fig. 2. In the CW case, the signal was generated by a Hewlett Packard (HP) 8690B Sweep Oscillator with an HP 8699B plug-in, filtered by an HP 360B low pass filter to remove harmonics, amplified by an Amplifier Research 4W1000 amplifier and a Varian VZL-6943G1 TWT amplifier and then filtered

again to remove harmonics and noise generated by the TWT. The power was adjusted to the desired level by setting the output level from the sweep oscillator. In the Pu case, the signal was generated by an Epsco PH40K and passed through a PAMTECK UTA1017 isolator to protect the pulsed source from excessive reflected power. In either case, the signal was passed through a coax-to-waveguide adaptor (Dielectric Communications C-42061-501). A reflectometer (Dielectric Communications D-40588-502) was used in conjunction with HP435A power meters to monitor forward and reflected powers. The impedance match to the lens holder was done using a triple stub tuner designed and built at Walter Reed. Typically, it was possible to adjust the tuner so that the reflected power was 15-18 dB below the forward power with the lens holder in place. The lens holder was located 1/4 guide wavelength from the waveguide shorting plate placing the lens holder in the maximum of the electric field. The vertical position of the lens holder was adjusted so that the lens was approximately at the center of the waveguide.

b) Microwave Exposure Conditions

Average transmitted powers were 60W, 20W, 6W and zero W for the shams. The corresponding SARs (as determined by measuring temperature elevation at the lens site with the collant flow off) were 1.0-1.2 W/g, 300-400 mW/g, 100-120 mW/g and zero respectively. Initially, the bath offset temperature to meet the final temperatures was 1.4-1.6^{o}C at 60W of average power (with coolant flow), 0.3-0.4^{o}C at 20W and very close to zero at 6W. The bathing medium was PBS. Flow rat was 600 ml/minute. More recently modifications to the circulating coolant system have permitted reductions in the offset temperature required to approximately 0.3^{o}C at the highest SAR with proportional reductions at lower SARs.

In the pulsed case, average power was set by variation of the pulse repetition rate. The peak power and pulse rise time were constant at about 20 KW and 0.1 to 0.5 sec, respectively. The pulse width used routinely was 10 sec. optimum for thermoacoustic expansion.

Lenses were fixed either immediately after irradiation or after the 2-day incubation in M199. After treatment with Karnovsky's fixative for 48 hours at 4^{o}C, the lenses were transferred to 0.1 M Na-cacodylate buffer, dehydrated in an alcohol series to acetone and critical point dried from CO_2. Each lens was quartered and attached to an SEM stub with silver daube paint, spatter-coated with gold-paladium and examined in a Hitachi HHS-2R SEM at an accelerating voltage of 20 KV.

c) High Voltage Electron Microscopy of Tissue Culture and Lens Fiber Cells

(i) Tissue Culture

Tissue culture cells are grown on gold mesh 30 mm grids to avoid any toxicity (Porter and Wolosewick, 1977). Grids are sandwiched between a formvar support film and a glass cover slip, and coated with a thin layer of evaporated carbon. These coverslips are sterilised under ultraviolet light and then used for cell cultures. After incubation, the grids are fixed in 2.5% glutaraldehyde in 1 M cacodylate buffer pH 7.2 for 20 minutes, rinsed briefly in the buffer and post-fixed in 1% osmic acid in cacodylate buffer for 1 hr. They are stained with unanyl acetate and lead citrate as for regular transmission electron microscopy. The grids are then carefully removed and transferred to a holder for critical point drying after dehydration in alcohol. The grids are examined in HVEM at the

University of Colorado at Boulder, Colorado.

(ii) Individual Lens Fibers

Individual fibers from the equatorial region of rat lenses fixed in Kainovsky's fixative were prepared by gentle teasing and placed on special sandwich-type grids for examination by HVEM in the facility at Boulder, Colorado.

(iii) Non-fixed Specimens

For examination by x-ray analysis, samples are frozen and lyophilized prior to examination, or frozen and prepared by camphene substitution and sublimation as described by Watters and Buck (1971). Subsequently such samples may be viewed using an SEM equipped with an x-ray analysis attachment (EDX) to establish concentrations of metal ions in the specimen. Further studies would be indicated if these tests reveal significant changes in concentrations for sodium, potassium, calcium, and magnesium in these cell layers. In addition to biochemical tests, these tests might include localization and staining of calcium and other divalent cations combined with transmission electron microscopy (Appleton and Morris, 1979; Zechmeister, 1979; McGraw et al 1980). Unfortunately these techniques lack sensitivity and are presently used mainly in calcifying systems and must still be combined with x-ray energy dispersion analysis. Three instruments permitting finer localization and direct determination of the concentrations of ions in tissue sections are (1) the CEDX scanning electron microscope equipped with x-ray emission detector (EDAX or KEVEX) and cryological stage which we intend to lease, (2) scanning transmission electron microscope (STEM) and (3) the secondary ion mass spectrometer (SIMS) in which ionic concentrations small sections of tissue can be determined by x-ray analysis and mass spectrometry respectively (Burns and File, 1980). At present a STEM is located near us (70 miles drive) at McMaster University and locally, under the aegis of the Second Century Fund of the University of Western Ontario, it is hoped to have an STEM located at Western in approximately two years. A SIMS is available at a U.S. Defence Department facility, The Naval Weapons Support Center, Crane, Indiana. We would hope that on some of our trips to Washington, we could visit this facility on our return trip to London and examine some of our speciments on the SIMS. This would require that they would help us to obtain permission to use the SIMS.

d) Cryological-SEM-EDAX sample examination (CEDA)

Fresh lenses are frozen on a cryostage, using octanol to attach it to the cryostage, and freeze-fractured. The frozen fractured specimen is transferred on a cold stage cooled with liquid nitrogen to the SEM specimen chamber and examined. A suitable area is chosen for x-ray analysis and the spectrum recorded.

e) Histochemical Sample Preparation and Staining

Following fixation as described above, or in Heidenhein's SUSA fixative (Gabe, 1976), the tissues are passed through 2 changes of 70% alcohol (12 hours), 2 changes of 95% alcohol (1 hour each), 4 changes of 100% alcohol (1, 1, 2 and 2.5 hours), 2 changes of xylene (2 and 2.5 hours) and 2 of wax (1 hour each).

f) Water-Soluble Embedding Method for Light Microscopy

It is difficult to cut large areas of lens for light microscopy when using conventional embedding media for TEM. This problem has been overcome by a commerc-

ially available methacrylate embedding kit. It consists of solution A containing hydroxyethyl methacrylate, polyethelene glycol and water; a catalyst, benzoyl peroxide; and solution B containing N, N-Dimethyl amiline and polyethelene glycol.

Fixed lens are quartered lengthwise to include the anterior and posterior poles and dehydrated in graded series of alcohol, 2 lens each, up to 95% ethanol. The tissue is transferrred to solution A containing 0.9% catalyst and allowed to infiltrate overnight. The lens pieces are suitably oriented in plastic capsules before pouring the embedding medium consisting of solution A + catalyst: Solution B in ratio of 20:1. The blocks, hardened at room temperature, are cut on dry glass knife, 1-2 sections are floated on water, dried on hot plate and stained with toluidine blue or hematoxylin and eosin.

g) Biochemical Techniques

In general, biochemical assays are performed on homogenates of the lens. In some cases special homogenizing media are used. In general either enzymic, spectrophotometric or spectrofluorometric, luminescence or bioluminescence, or HPLC (high performance liquid chromatography) techniques are used. Because of the large number of assays, pertinent information is summarized.

Protein

extract: homogenize in water or 8 M guanidinium chloride
method: Biorad or Lowry
sensitivity: 1-5 g protein.

Adenylate Nucleotides

extract: homogenize in 8 M guanidinium chloride
method: Luciferin-luciferase bioluminescence
special equipment: Lumac Biocounter coupled to Commodore PET computer
sensitivity: 10^{-15} moles ATP

NADPH, NADH

extract: homogenize in 8 M guanidinium chloride
method: bioluminescence using Lumac kit
special equipment: Lumac biocounter coupled to Commodore PET computer
sensitivity: 10^{-12} moles ATP

Superoxide anion

extract: 8 M guanidinium chloride
method: Luminol luminescence
special equipment: Lumac biocounter coupled to Commodore PET computer
sensitivity: unknown at present, probably similar to ATP

Glutathione

extract: (a) 10% Trichloroacetic acid
(b) 8 M guanidinium chloride, then made 90% in alcohol to precipitate chloride
method: (a) enzymatic cycling assay (Riley and Yates, 1977)
(b) HPLC using special resin

special equipment: (a) recording thermostatted spectrophotometer
(b) HPLC (equipped with polarographic detector which we currently do not have)
sensitivity: (a) 10^{-12} moles
(b) 10^{-6} moles

Vitamin E

extract: homogenate, extracted with hexane method: (a) spectrofluorometric (b) HPLC with UV or fluorescence detection
special equipment: (a) spectrofluorometer
(b) HPLC with UV or fluorescence detector
sensitivity: (a) 1-10 g
(b) 0.05 g using UV detector; expected improvement of 100 fold using fluorescence as compared to UV detector

Vitamin C

extract: homogenate using either 8 M guanidinium chloride or 10% Trichloroacetic acid
method: HPLC using amino column with UV detector
special equipment: HPLC
sensitivity: unknown at present, expect 10 g detectable conveniently

NEW RESULTS: (a) High Voltage Electron Microscopy Investigations of Cytoskeletal Changes in Tissue Culture and Single Equatorial Lens Fiber Cells

Since we have previously suggested that changes in cytoskeletal structure might be involved in the process of globular degeneration (Mousa et al, 1979), it seemed appropriate to investigate these cytoskeletal changes by High Voltage Electron Microscopy (HVEM). Using as a model the reversible changes in morphology termed "arborization" induced by cytochalasin D in our RLE-R line of cells (derived from the rat lens by Miller et al, 1979), Dr. Sanwal showed that corresponding disorganization of the cytoskeletal and formation of thick filaments occurred coincident with the formation of globules by the cell surface (please see appendix).

In her first attempt to examine isolated fixed single lens fiber cells, Dr. Sanwal encountered some difficulty with instability of some of the specimens, in the high energy beam of the instrument. The structure of the cytoskeleton in a fiber cell obtained from a rat lens which had been exposed to a cataractogenic elevation of temperature was examined. Although no satisfactory control was available, the cytoskeletal structure visible was similar to that induced by CD treatment of culture RLE-R cells. These data are consistent with the idea that similar cytoskeletal changes are involved in the globular degeneration observed in CD- and heat-induced cataracts.

(b) High Resolution Light Microscopy

The 1 -2 sections obtained using this technique have made possible the routine use of light microscopy as a technique for the lens. Previously, because of its high protein content, the hardness and brittle sections obtained from the dehydrated lens had necessitated elaborate dehydration and/or embedding procedures specially designed for the lens. Examples of these sections obtained using

this technique are shown in Fig. 6 for (a) control lens, (b) lens irradiated with CW microwaves which has apparently normal morphology, and (c) lens irradiated with Pu microwaves.

(c) Preliminary Investigations of Reciprocal Relationship Between Dose Rate and Time Required for a Defined Amount of Damage

The depth of degeneration observed after a 48 hr incubation in M199 at 55.5°C appeared to increase in response to increasing microwave dose rate. For exposures conducted for the same time period, either 5 minutes or 20 minutes. When the actual total dose was used as a basis for comparison, depth of degeneration was very similar in samples which received equal or similar doses - for instance: (1) 20W for 5 min (0.6 kjoules) and 6W for 20 min (0.6 kjoules); or (2) 60 W for 5 min (18 kjoules) compared to 20 W for 20 min (24 kjoules) showed similar depths of globular degeneration.

(d) Preliminary Cryo EDX Results

In Figure 9a is shown a preliminary (Hitachi) SEM picture of the surface of a lens incubated in elevated glucose. This was obtained after freeze-fracturing using the cryogenic stage. The lens fiber cells and foamy area of the cataractous lens appear to be distinguishable from each other in this fresh specimen. The EDX scans of these two areas (fibers and foam) using the semi-quantitative EDX show apparent differences in elemental composition. The solid (filled in) curve corresponds to the normal lens fiber cell area, while the diseased area (white line) apparently has lower concentrations of sodium, chloride and elevated calcium. Since it has been suggested that elevation of calcium may contribute to cataract formation, the preliminary indications are consistent with current thinking in this area, and point to several important possibilities for extension of the experiments if this machine is obtained.

(e) Development of Biochemical Assays

Vitamin E

Although in our initial studies with diabetic rats fed diets with additional Vitamin E, we extracted the serum Vitamin E with hexane and estimated it by fluorescence, more recently we have been using the more modern technique of high performance liquid chromatography developed by Bieri's group (1979). Although this technique has been satisfactory for standards, and for analysis of medium to which Vitamin E was added, it was not sufficiently sensitive to detect any Vitamin E in lenses by ultraviolet absorption. Because of this we are upgrading the HPLC detection system to permit fluorescent detection of the Vitamin E which is approximately one hundred times more sensitive than the present method of ultraviolet detection. Once this equipment has arrived we hope to proceed with analyses of batches of lenses and of samples.

Vitamin C

This assay is been developed for use on samples of serum, aqueous, vitreous and lens since it has been reported that Vitamin C falls rapidly as one of the first steps in microwave cataractogenesis. Dr. Stewart-DeHaan is developing techniques using HPLC analysis, for determining the concentrations of this important antioxidant vitamin in such biological fluids. In initial experiments she has

used the Waters amino column with sodium phosphate buffer, with satisfactory results for standards (not shown). Further work on actual samples is planned.

Determination of ATP

ATP is required for maintenance of the normal cytoskeletal structure, in particular the actin microfilaments whose disruption by agents such as CD apparently results in globular degeneration of the lens. Any changes in ATP level may thus be linked to globular degeneration.

ATP is extracted, ATPases denatured and protein determination simplified by homogenization of lenses in 8 M guanidinium chloride using a Polytron homogenizer.

Aliquots of the homogenate are diluted 1:100 to reduce the level of guanidinium chloride to non interfering levels and 100 l taken for analysis using the Lumac Luciferase-Luciferin assay kit (available in Canada through Mandel Scientific Co.). This assay utilizes a Lumac Biocounter (Lumit, available from Mandel Scientific Canada) which we are presently coupling to a Commodore PET computer to automate recording of data. Results obtained to date indicate that significant decreases of lenticular ATP levels occur after 24 hr incubation in M199 (80% reduction). Cataractogenic treatments, such as exposure to elevated temperature (41^{o}C for 20 min) or elevation of glucose level in M199 to 55.6 mM (10 x normal), further decrease the level of ATP to approximately 1/2 of the concentration in incubated controls. It is planned to extend these studies to the individual areas of the lens which show the first signs of damage after exposure to cataractogenic conditions; in particular, the lens equator and epithelium will be examined, in order to ascertain whether these suffer an earlier change in ATP levels than the other areas of the cortex in which cataractous changes occur later.

Glutathione

Glutathione (GSH) is necessary for the maintenance of many cellular proteins which have sulfhydryl groups. In particular, it is postulated to be necessary for the maintenance of the integrity of the cell membrane of many cell types, including red blood cells, and the lens cells. It is extracted by aqueous buffers containing ethylenediamine tetraacetic acid (EDTA) a chelating agent for metal ions, in order to prevent formation of oxidized glutathione (GSSG). We have used the techniques for analysis of glutathione developed by Riley and Yates (1977) based on enzymic reduction of the Ellman reagent DTNB by a cycling assay using NADPH and glutathione reductase. Preliminary experiments have been satisfactory, but an attempt to automate the assay ran into several technical problems; this has resulted in efforts towards this aspect of the analyses being deferred in favour of development of a chromatographic technique using HPLC.

Pyridine Nucleotides NADH and NADPH

These nucleotides, in particular NADPH, appear to be very important for maintaining cell membrane integrity in both the lens and red cell, because of the role of NADPH in reducing oxidized glutathione by way of the enzyme glutathione reductase. NADPH levels are known to fall markedly just prior to the stage of opacification in x-ray cataracts.

Levels of NADPH and NADH may be measured as low as 10^{-12}M by a bioluminescence assay available from Lumac, which enables very low concentration of these

nucleotides to be determined using the Biocounter. Because of the low levels which may be determined, it is expected that it will be possible to use the same guanidinium chloride extracts as used for ATP determination (and below, superoxide-radicals), and to combine this with dissection of particular areas of the lens, in order to investigate initial precataractous changes in the lens.

Detection of Superoxide Free Radicals using Luminol Assay

In the literature it has been suggested that various free radicals and activated oxygen species including superoxide free radicals, hydrogen peroxide, singlet oxygen and hydroxyl radicals may be involved in oxidative damage to the lens, which results in membrane damage and cataractous globular degeneration.

Of these, superoxide may play a major role, since the other species usually are formed from it. We have estimated superoxide radical conveniently using the Lumac Biocounter, and expect to use this to assay for levels of superoxide dismutase in the lens.

Using this technique, levels of superoxide in a guanidinium chloride extract have been found to increase markedly during the course of a 24-hour incubation of rat lenses in M199 or M199 plus glucose.

PREVIOUS RESULTS: (a) Thermal Controls/Elevated Temperature

Previous results by SEM:

In a previous progress report, we indicated that, when unirradiated lenses were exposed to various temperatures for 1 hour, and then incubated at 35.5° C for 2 days in M199, then fixed, the transparency of the lens decreased progressively as the temperature of incubation increased, in the range of 37-50°C. However, after incubation at 60° C, no loss of transparency could be seen. Examination of the lenses by SEM revealed, at 37° C, no changes in the normal morphological features of the lens epithelial or fiber cells. At 39° C, the equatorial epithelial cells were not tightly joined to each other at the cell borders, which appeared swollen, were curled up from the underlying fiber cells, and had pitted surfaces; also at 39° C, equatorial fiber cells showed decreased interdigitation; in one area they were twisted and distorted and, at the equatorial region itself, showed incipient globular degeneration in a wedge-shaped ring (with the wedge apex towards the lens nucleus). At 41° C the lens epithelial cell surfaces were fibrous and necrotic, with many deep pits, and in many areas the underlying fiber cells were denuded of their epithelial coating; the fiber cells were extensively involved in globular degeneration which consisted of a much larger equatorial, wedge-shaped area (at which the maximum depth of globular degeneration was measured), and which now extended anteriorly and posteriorly to the poles of the lens (where a minimum depth of degeneration was measured). Pre- and post-incubation in vitamin E enriched medium prevented most of the globular degeneration associated with exposure to 41° C. At 43° and 45° C, no epithelial cells *per se* could be identified, and the depth of degeneration increased progressively.

An unusual feature seen first at 47° C and 50° C was the appearance of very large globules (up to 200μ in diameter, 10 times the usual size). At 60° C there were no apparent differences in morphology from normal lenses, either in epithelial or fiber cells, presumably because the cells had been "fixed" by the extreme temperature. Only slight changes in morphology of nuclear lens fiber cells were

detectable in lenses incubated at 45° C, as compared to those at 37° C. These data are consistent with our previous suggestion that globular degeneration and opacity appear to be associated in a causal sense. The absence of globular degeneration in lenses exposed to 60° C, which results in heat fixation, appears to account for the lack of lenticular opacity.

(b) Microwave Exposure

Exposure to Pu irradiation was performed under conditions optimized for thermoacoustic expansion (within the capabilities of the EPSCO PH40K pulsed source). Thermoacoustic transduction was verified by a third octave analysis of the radiated sound field. Such Pu radiation (Fig. 3a, b) resulted in more extensive damage to lenses than CW radiation at the same average power or equivalent temperature exposure at all temperatures tested. Even at 37° C where no change in morphology was observed in unirradiated samples, damage could be detected at SARs delivered to the lens of as little as 120 mW/g. The amount of damage appeared to increase roughly in proportion to the SAR and/or time of irradiation, a relationship which suggests reciprocity but which requires further testing. Features of the damage observed following Pu irradiation were: (1) the appearance of holes in the immediately fixed, critical-point-dried lens fiber cells irradiated by Pu microwaves at temperatures as low as 37° C (Fig. 4a, b); (2) more extensive globular degeneration than observed in thermal controls at 39° C and 41° C for the same duration at identical temperatures; and (3) the appearance of large globules in the microwave irradiated lenses at 37° C, similar to those observed only at temperatures of 47° C and 50° C in unirradiated samples.

In order to ascertain if the damage to the lens required the 2-day incubation for detection by SEM, several lenses were fixed immediately after Pu irradiation. Such lenses showed rather large numbers of holes, which was not noted for CW; these were similar in number to the holes found in the lenses incubated for 2 days following the irradiation. A similar analysis of the temperature controls where fixation took place immediately after exposure to elevated temperataure, did not reveal the presence of holes in the lens fibers.

Discussion

In considering the possible hazards of microwave irradiation for military personnel, it is appropriate to consider not only the effects of acute damage which might develop following inadvertent exposure to high doses under combat conditions, but also possible long-term effects which might ensue following low level exposures over a longer period of time. Although such experiments could be conducted in a living animal, it appeared advisable, in beginning these experiments, to use a more sensitive system in which the [illegible]aractogenic response occurs rapidly and large doses which would ensue that a me[illegible] effect was found. Threshold studies will be conducted later once an appropr[illegible] to check is established.

The [illegible]stem developed for exposing lenses in medium to the stress of chemica[illegible]nds, temperature, and irradiation permits the precise manipulation of these f[illegible]tors, whereas, in vivo, only an approximation is possible. In addition, the various types of damage and their degree or extent are more readily and rapidly detectable and assessable; for example, incubation with 55.6 mM glucose in M199 results, within 2 days, in development of both globular degeneration and opacity, while the same process in the intact diabetic animal would require as much as 6-8 weeks to reach a similar degree of degeneration (accepted for pub-

lication).

Several observations in lenses exposed to elevated temperature alone are pertinent to and useful in the interpretation of the effects of microwaves on lenses incubated in a circulating medium of constant temperature. (1) the linear relationship between maximum depth of globular degeneration and temperature up to 50° C (Fig. 5) will be useful in interpolating additional effects of microwave irradiation on the isolated lens in terms of temperature equivalence. (2) The large globules found in microwave irradiated lenses at 37° C and in unirradiated lenses at 47° and 50° C are another morphological marker found in our studies of lenses incubated in vitro (only in lenses exposed to elevated temperature, but never in lenses exposed to other cataractogenic conditions, as diverse as elevated levels of sugars, steroids, hygromycin B or drugs such as cytochalasin D, colcemid, or vinblastin sulfate). This observation should also be useful in estimating the "temperature elevation equivalent effect" of additional microwave irradiation. (3) The observation that incubation with vitamin E-enriched M199 prevented most of the cataractogenic globular degeneration in lenses heated to 41° C, indicates a possibility for both prevention of potential heat-induced cataracts and a rationale for treatment of individuals whose eyes are inadvertently exposed to large amounts of heat and/or infrared radiation. It is presently unclear whether the vitamin E acts as an antioxidant or scavenger of free radicals or on some other membrane activity such as fluidity. Preliminary observations on lenses exposed to Pu microwave radiation indicate that high energy pulsed radiation can apparently cause an additional effect - holes in the cell membranes observed after fixation and critical point drying - when compared to unirradiated controls at the same temperature. This effect appears to occur either concurrently with the irradiation or immediately afterwards, since it is found both in lenses fixed immediately after Pu irradiation as well as after the further 47 hour incubation. No SEM detectable defect was observed with temperature elevation alone without the 2-day incubation in M199.

Thermoacoustic expansion (Foster and Finch, 1974) at 37° C apparently results in large globules, an effect equivalent to a 10° C temperature elevation.

Comparison of the results of CW and Pu irradiation indicates that CW was not as effective as Pu in causing damage to the lens, possibly because of the additional mechanical stress resulting from thermoacoustic expansion caused by Pu microwaves. In that context, it is useful to recall the fact that adjacent fibers are mechanically connected by numerous regions of interdigitation along their lengths. Further, the thermal conductivity and specific heat of the lens fibers is anisotropic due to its large ratio of length to width and the different constitution of its materials in length (cytoplasm, cytoskeleton) and width (membrane). Thus, mechanical amplification can take place as would be the case with a bimetallic strip. CW irradiation did, however, also result in extensive globular degeneration in lenses incubated 2 days following as little as 5 minutes at maximum SAR at 37° C, a temperature at which no degeneration would have been expected in the absence of microwaves. Microwave exposures at elevated temperatures for both CW and Pu radiation resulted in more extensive lenticular damage than irradiations at 37° C.

Special notice should be taken of the apparent proportional increase in depth of globular degeneration, as a function of dose rate, and the similar extent of damage observed for different SARs at times which resulted in a similar total dose being delivered to the sample. Although the possibility of reciprocity, generally

recognizes a feature of ionizing (but not of nonionizing) irradiation, has not been suggested to date for microwave irradiation, these data suggest that further studies of pulsed irradiation should consider this. If also true in vivo, such reciprocity could have important implications for long term chronic exposure to low levels of pulsed microwave irradiation. This would imply that long term exposure to a low level of pulsed microwave irradiation, such as might occur in military personnel working in the vicinity of a radar installation, might result in increased risk of cataractogenesis.

The mechanism of cataractogenesis in the isolated lenses following exposure to microwave irradiation is still not well-defined. Nevertheless, certain features of this process have been suggested by the experiments on high voltage electron microscopy reported above. The peculiarly similar appearance of the cytoskeleton in microwave-irradiated fiber cells, and tissue culture cells treated with cytochalasin D, is consistent with the idea we have presented previously, that in cortical cataracts the critical change leading to globular degeneration and opacity is a change in the cell cytoskeleton (Mousa, Creighton and Trevithick, 1979). Further work which needs to be done to confirm this and to define the biochemical and physical changes (i.e. in ATP, Na/K ratios and levels, NADPH and glutathione) etc. which may be responsible for this cytoskeletal change, is proposed in an accompanying application for continuation of this grant.

The biochemical results on cataractogenis in vitro induced by heat, microwaves and glucose are consistent with several possible mechanism for globular degeneration:

(1) The lowering of ATP levels would be expected to result in supercontraction of the actin microfilaments.
(2) The increased level of superoxide radicals with time would be expected to result in oxidative damage to sulfhydryl groups, or oxidation of unsaturated fatty acids, and proteins of membranes, as might the previously reported (by Kinoshita's group) decreased or of reduced pyridine nucleotide or glutathione, reported for radiation cataract recently by Reddy's group (Giblin) level of Vitamin C.
(3) A decrease of potassium/sodium ion ratio, which might result from leaky cell membranes, would be expected to cause a change in contraction of cytoplasmic microfilaments, since potassium is required for the process of microfilament contraction.
(4) The possibility of some prostaglandin, or thromboxane, or prosacyclin metabolite generated to a larger extent acting on a cell membrane or cytoskeletal receptor; this of course would be reduced by biological antioxidants such as BHT, glutathione and vitamin E.

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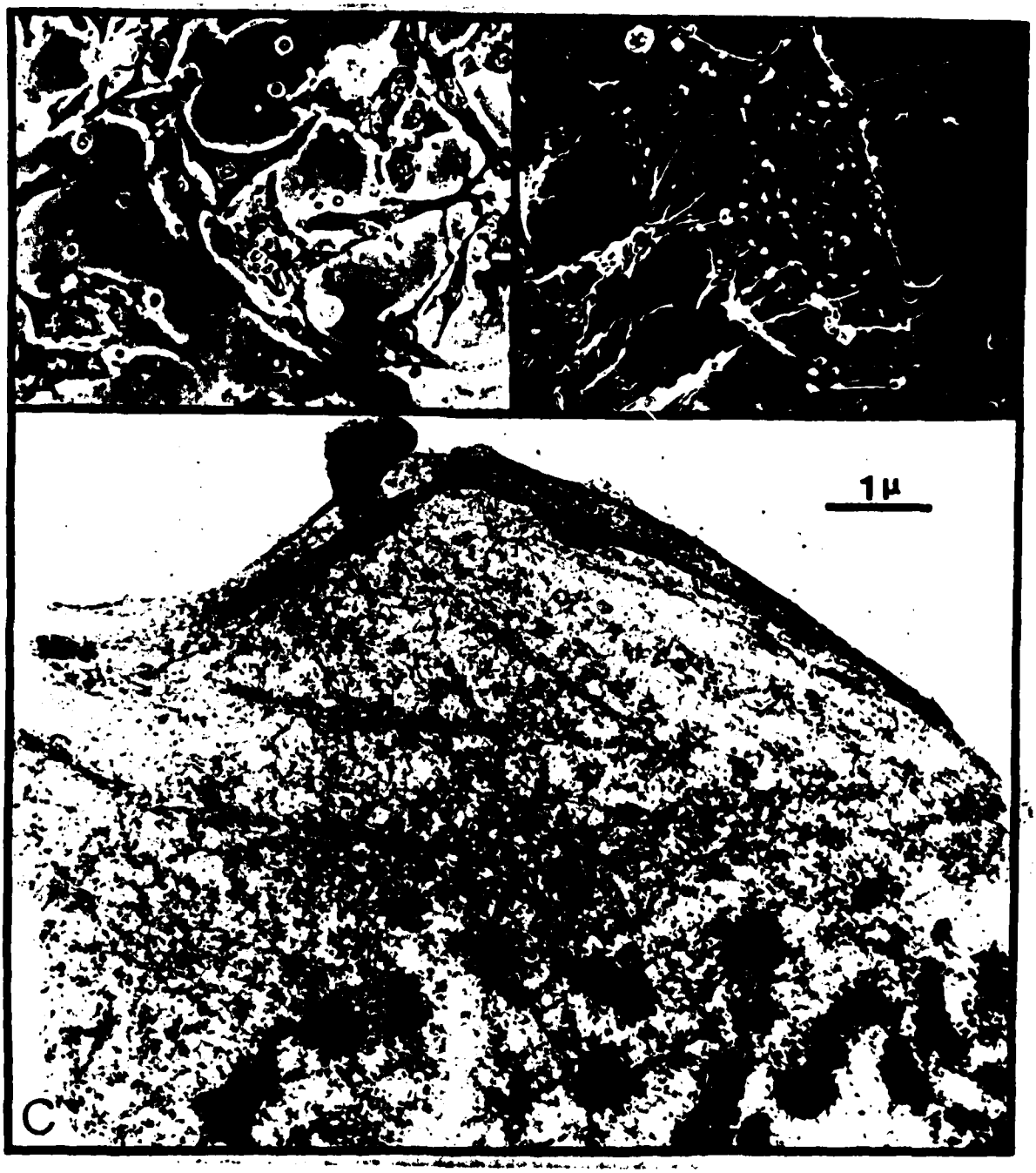
1μ
C

Figure 1: Phase Contrast Microscopy, Scanning and High Voltage Electron Microscopy of RLE-R Line of Rat Lens Cells Growing in M199. Note organization of microtrabecular network especially at cell periphery.

A. 3 day old control cells. x600.

B. Scanning electron micrograph of control cells showing normal surface morphology. x 2,500.

C. High voltage electron micrograph of a control cell showing distinct arrays of microfilaments under the cell membrane. x 18,600.


1μ
A
1μ
B

Figure 2: Phase Contrast Microscopy, Scanning and High Voltage Electron Microscopy of RLE-R Line of Rat Lens Cells growing in M199 after 60 min in 10^{-6} M CD followed by 60 min. in normal medium. Note reappearance of microtrabecular meshwork at cell periphery.

A. General view of a cell with numerous stalk-like processes and blebs. x 8,250.

B. Part of cell with many cellular processes. Note the absence of continuous microfilament bundles under the cell surface. x24,500.


A
10μ
10μ
0,5 μ
C

Figure 3: Phase Contrast Microscopy, Scanning and High Voltage Electron Microscopy of RLE-R Line of Rat Lens Cells growing in M199 after 60 min. in 10^{-6} M CD followed by 60 min. in normal medium. Note reappearance of microtrabecular meshwork at cell periphery.

A. The cells show a lack of arborization. x600.

B. Scanning electron micrograph shows that most of the cells have normal surface appearance. x650.

C. HV electron micrograph of a reverted cell. There are no cellular processes or surface blebs. Note the reorganization of filament bundles. Some of them are diverting inwards. x33,000.

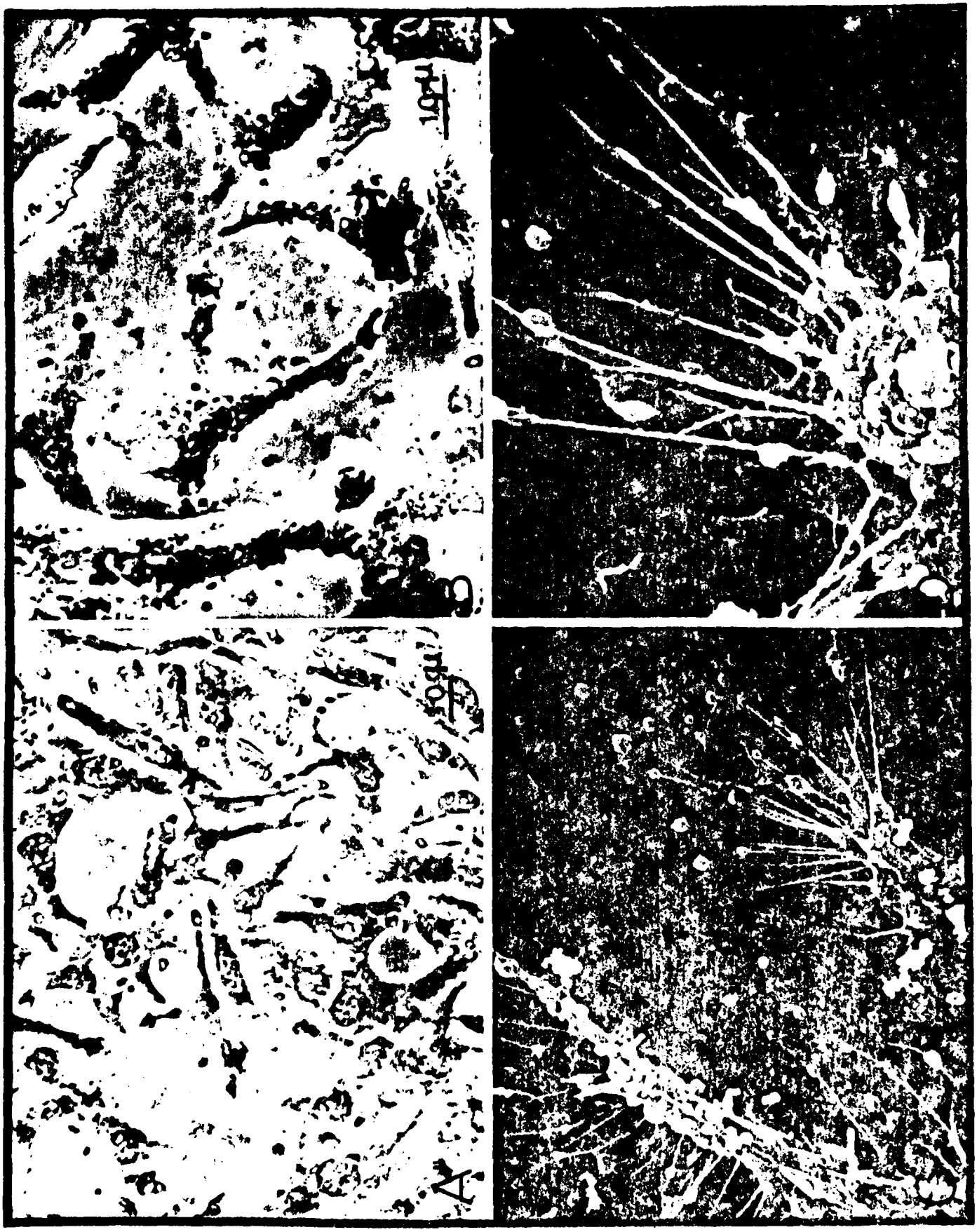
10μm
10μm

Figure 4: Effect of CD (10^{-6}M) On the Cell Line

A. 60 minutes after CD treatment cells show arborised appearance. x 700.

B. Similar cells showing surface blebbing. x 1,130.

C. Scanning electron micrograph showing a part of the arborised cell with numerous stalk-like processes and blebs. x3,000.

D. Scanning electron micrograph of a cell with an aggregation of blebs on the surface. x8,000.

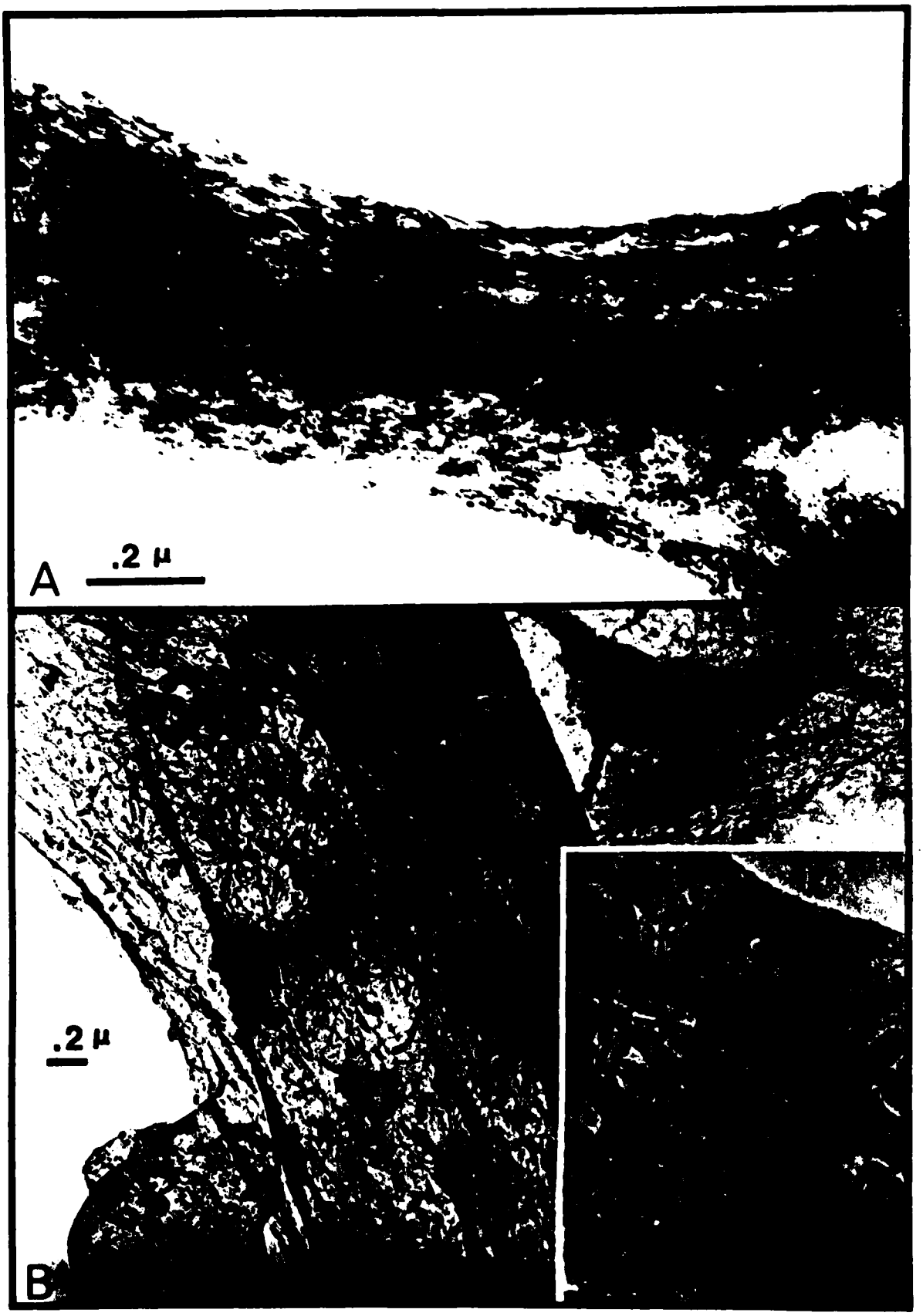
A
.2 μ
.2 μ
B

Figure 5: High Voltage Electron Micrographs of Single Lens Fiber Cells from Rat

A. dissected cortical fiber from a heat treated lens. Dark stained microfilaments are visible x 150,000.

B. high voltage electron micrograph of dissected nuclear fibers from a heat treated (41^{o} C, 1 hr) and glycerinated lens. No cytoplasm is seen, but the filaments are very prominent. They appear somewhat disorganized, when compared to tissue culture cells, although a normal control for the lens fiber cells must still be obtained.

C. Part of B further magnified x 200,000.

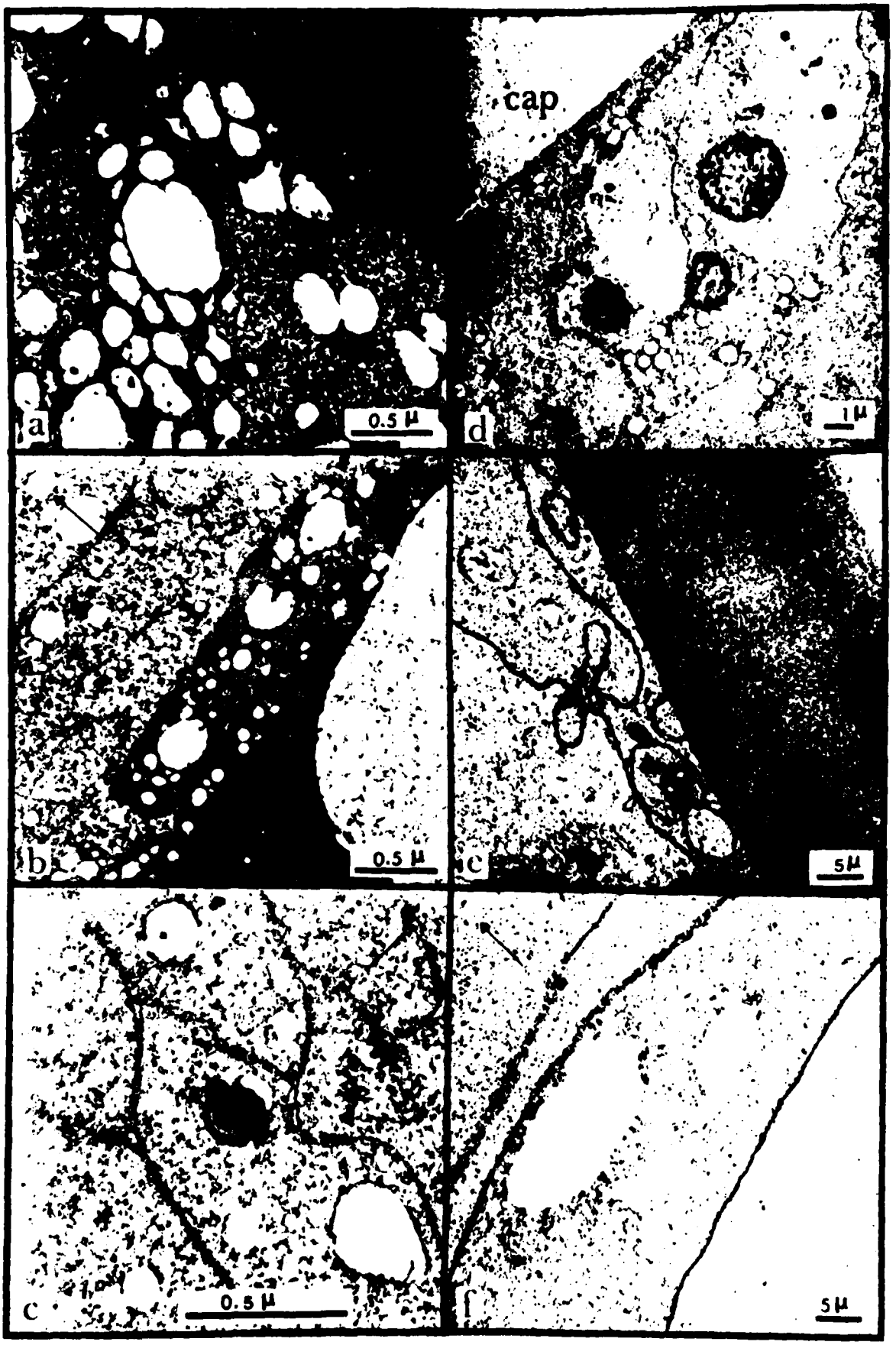
a
0.5 μ
cap
d
1μ
b
0.5 μ
c
5μ
e
0.5 μ
f
5μ

Figure 6: Electron Micrographs of Microwave Radiated Lenses. a.b.c. Pu/65W/ 20 min/37°

a. Shows the capsule and the epithelial cells filled with numerous vacuoles, the larger ones may be derived by fusion of smaller vacuoles x 50,000.

b. Superficial cortical fibers shows vacuoles of various sizes x 50,000.

c. Deeper cortical fibers show fewer vacuoles; the vacuoles appear to have a distinct membrane. Myelin figures are commonly seen x 100,000.

d.e.f. cw/65W/6 min/39°/QF.

d. Capsule and some epithelial cells. A newly differentiating cell shows many vacuoles x 9,000.

e. Fiber cell at the postequatorial zone have an undulating membrane as compared to normal cells x 28,000.

f. Deeper cortical cells show some vacuoles x 28,000.

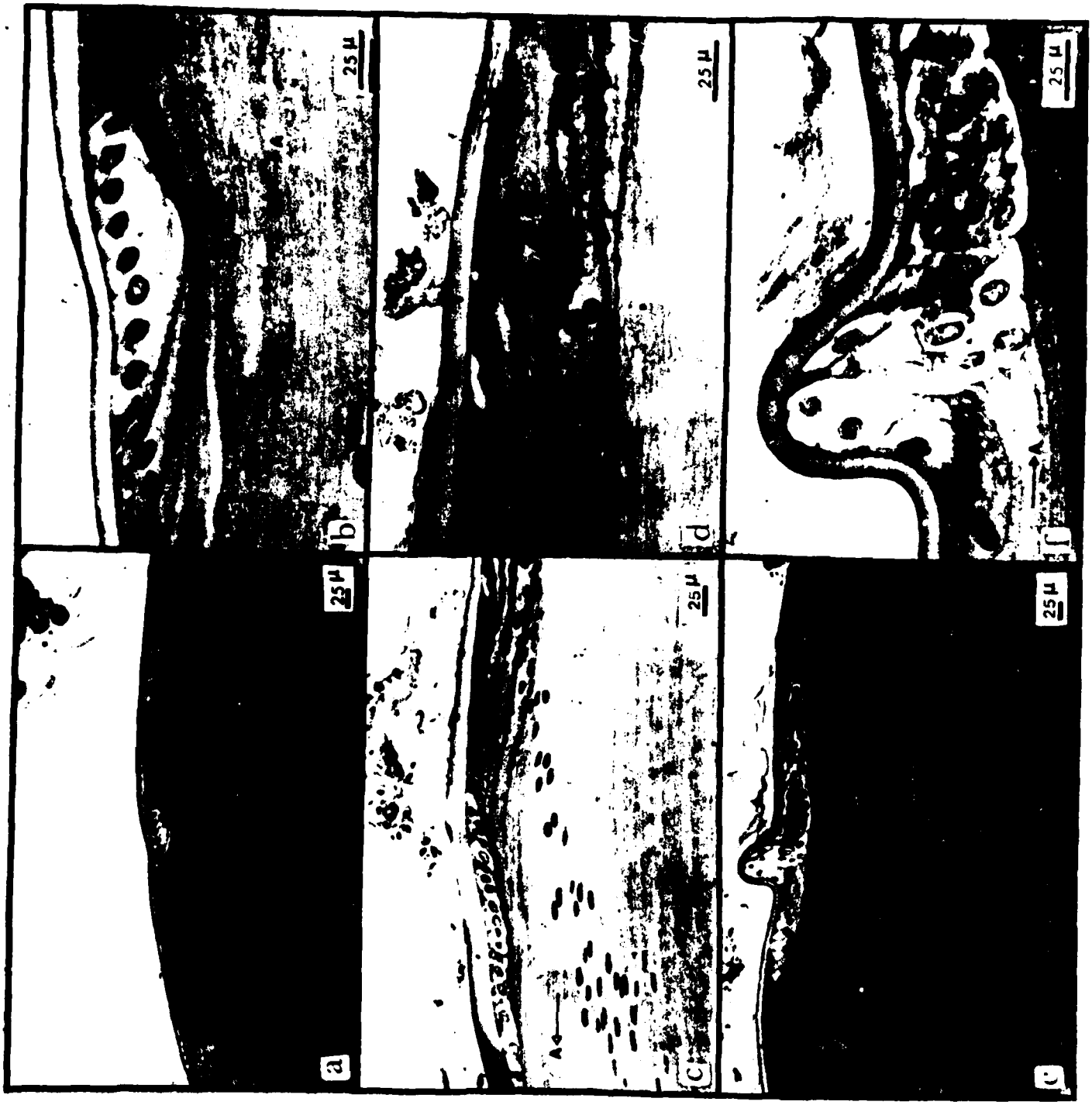
a
25 μ
b
25 μ
c
25 μ
d
25 μ
e
25 μ

Figure 7:
a.b. Light microscope sections of polmethracrylate JB-4 embedded lenses Fresh lens. Section through equatorial region shows well organized lens fibers, the epithelial layer and the lens capsule which is thicker in the preequatorial region a x 250, b x 800.

c.d. Microwave irradiated lens. cw/65W/6 min/39°/Quick FK. In the post-equatorial zone the newly joined lens fibers are less regularly arranged and appear c x 400, d x 800.

e.f. Microwave radiated lens, Pu/65W/6min/39°/Quick FK. Equatorial and preequatorial zone shows multilayered epithelial, perhaps due to cell migration from the adjoining region or contraction of the group of cells into a cellular plaque e x 250, f x 800.

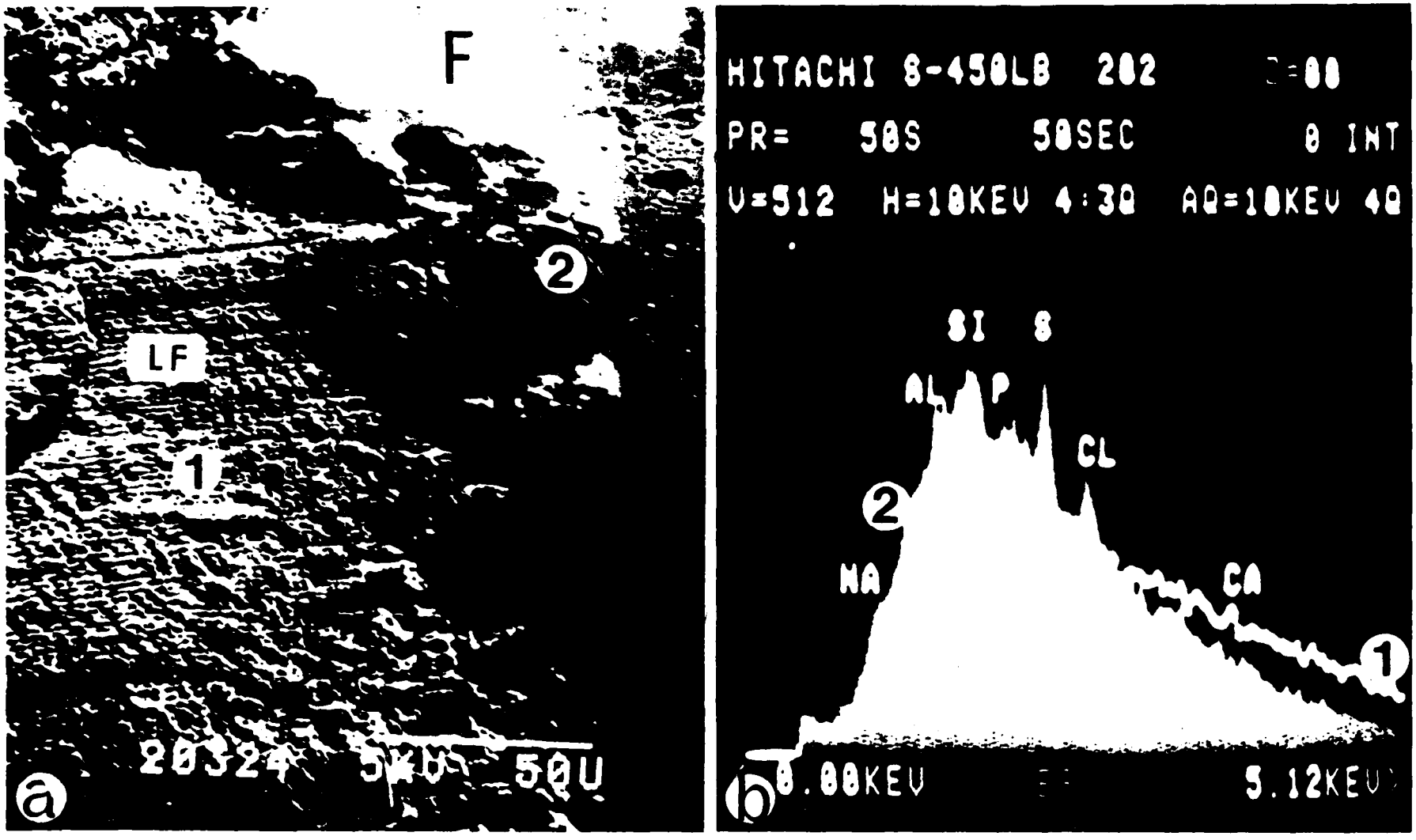
F
LF
1
2
a
HITACHI S-450LB 202
PR= 50S 50SEC 0 INT
V=512 H=10KEV 4:30 AQ=10KEV 40
SI S
AL P
CL
2
NA
CA
1
b
.00KEV
5.12KEV

Figure 8: Cryological SEM EDX detection of ion levels in lens made cataractous by 24 hr incubation in M199 with elevated glcuose. The lens was frozen and freeze-fractured on the Hitachi cryological stage prior to examination. In (a) is shown the SEM picture of the lens equatorial area with lens fiber cells (LF) and foamy (F) cataractous area. In (b) are shown the Kevex EDX scans of these two areas 1 and 2 which are identified as 1 and 2 on the appropriate EDX scans.

TABLE 1

ATP Levels in Lenses Heated to 42° C for 20 min and Exposed to 55.6 mM glucose

Incubation Conditions	ATP ng/g wet lens*
Control Zero time	153
Control, Incubated 24 hr.	30.2
42° C (30 Min)	16.3
55.5 mM glucose	15.8

* Except for zero time controls which were frozen immediately, all lenses were incubated in groups of 3 and weighed. ATP levels were determined on homogenates of each lens as described in the text and triplicate averaged.

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