USAFSAM-TR-87-32

EFFECTS OF SIMULTANEOUS RADIOFREQUENCY RADIATION AND CHEMICAL EXPOSURE OF MAMMALIAN CELLS

Volume II

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July 1988

Final Report for Period 2 January 1984 - 31 December 1986

Approved for public release; distribution is unlimited.

Prepared for
USAF SCHOOL OF AEROSPACE MEDICINE
Human Systems Division (AFSC)
Brooks Air Force Base, TX 78235-5301

NOTICES

This final report was submitted by the Department of Radiology, the University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, Texas, under contract F33615-84-C-0604, job order 2312-W1-18, with the USAF School of Aerospace Medicine, Human Systems Division, AFSC, Brooks Air Force Base, Texas. Dr. David N. Erwin (USAFSAM/RZP) was the Laboratory Project Scientist-in-Charge.

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The animals involved in this study were procured, maintained, and used in accordance with the Animal Welfare Act and the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources - National Research Council.

The Office of Public Affairs has reviewed this report, and it is releasable to the National Technical Information Service, where it will be available to the general public, including foreign nationals.

This report has been reviewed and is approved for publication.

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UNCLASSIFIED
SECURITY CLASSIFICATION OF THIS PAGE

AD-A202 780

REPORT DOCUMENTATION PAGE

1a. REPORT SECURITY CLASSIFICATION Unclassified	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION / AVAILABILITY OF REPORT Approved for public release; distribution is unlimited.
2b. DECLASSIFICATION / DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S) USAFSAM-TR-87-32
6a. NAME OF PERFORMING ORGANIZATION Dept. of Radiology, University of Texas Health Science Center 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION USAF School of Aerospace Medicine (RZP)
6c. ADDRESS (City, State, and ZIP Code) 7703 Floyd Curl Drive San Antonio, TX 78284-7800	7b. ADDRESS (City, State, and ZIP Code) Human Systems Division (AFSC) Brooks Air Force Base, TX 78235-5301
8a. NAME OF FUNDING / SPONSORING ORGANIZATION 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER F33615-84-C-0604
8c. ADDRESS (City, State, and ZIP Code)	10. SOURCE OF FUNDING NUMBERS

PROGRAM ELEMENT NO.	PROJECT NO.	TASK NO.	WORK UNIT ACCESSION NO.
62202F	2312	W1	18

11. TITLE (Include Security Classification)
Effects of Simultaneous Radiofrequency Radiation and Chemical Exposure of Mammalian Cells: Volume II

12. PERSONAL AUTHOR(S) Meltz, Martin L.; Ciaravino, Victor; Kerbacher, James J.; and Eagan, Phyllis

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Final	FROM 84/01/02 TO 86/12/31	1988 July	85

16. SUPPLEMENTARY NOTATION

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	Chemical treatment of cells; in vitro exposure of cells; mammalian cells; microwave hazard; mutagenesis; and radiofrequency radiation
06	07		
14	02		

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

A circulating water-bath exposure system has been designed for in vitro radiofrequency radiation (RFR) exposure studies in the 915 to 2450 MHz range. A continuously rotating Styrofoam float, holding ten T-25 tissue culture flasks, averages out field heterogeneity and allows mixing in the medium in the flasks. Factors found to affect the specific absorption rate (SAR) in the medium include: 1) the position of the exposure flasks relative to the long axis of the antenna horn; 2) whether or not the flasks are exposed while stationary or in rotation; 3) the volume of the medium in the flask; and 4) the depth in the medium in the flask at which temperatures for SAR calculation are measured. The presence of cells in the exposure flask (as attached monolayer or cell suspension) did not result in an SAR different from that measured in the medium without cells present.

(Cont'd. on p. ii)

20. DISTRIBUTION / AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT. ☐ DTIC USERS	Unclassified
22a. NAME OF RESPONSIBLE INDIVIDUAL David N. Erwin, Ph.D.	22b. TELEPHONE (Include Area Code) (512) 536-3582
	22c. OFFICE SYMBOL USAFSAM/RZP

DD FORM 1473, 84 MAR 83 APR edition may be used until exhausted. All other editions are obsolete.

SECURITY CLASSIFICATION OF THIS PAGE
UNCLASSIFIED

19. ABSTRACT (Continued)

We have posed two hypotheses relative to the mutagenic activity of RFR: a) that RFR by itself is genotoxic to mammalian cells in vitro; and b) that a simultaneous exposure of mammalian cells to RFR during treatment with a genotoxic chemical will result in an alteration of the genotoxic activity of the chemical alone. For 4-hr pulsed wave RFR exposures at 2.45 GHz, 0.25 duty factor, and SARs in the vicinity of 30-34 W/kg, with the L5178Y mouse leukemic cell thymidine kinase locus mutation assay system being used, two conclusions were drawn from repeat experiments with both mitomycin C (MMC) and proflavin. These two conclusions were that: a) RFR exposure alone (at moderate power levels which resulted in a temperature increase in the cell culture medium of no more than 3°C) is not mutagenic and, b) during simultaneous treatment, the RFR does not affect either the inhibition of cell growth or the extent of mutagenesis resulting from the chemical treatment alone.

The same two hypocheses were explored for induction of sister chromatid exchanges (SCEs) and chromosome aberrations in Chinese hamster ovary cells which were similarly exposed to RFR and chemicals. The chemicals studied for SCE induction were MMC and Adriamycin. The results were the same after 2-hr exposures, similar to those described above: a) the RFR alone did not induce SCEs; and, b) no statistical differences were noted in SCE frequency, after simultaneous RFR exposure, from that induced by either chemical treatment alone. Likewise, 2-hr RFR exposure did not induce chromosome aberrations (in repeated experiments). In MMC-treated cells, no RFR-chemical interaction on chromosome aberration induction was noted. In contrast, in Adriamycin-treated cells, increased temperatures produced by either RFR or water-bath heating led to a very small overall increase in the total frequency of chromosome aberrations. This increase, attributable to temperature increase (rather than to RFR), is being further explored.

Keywords: Microwaves

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EFFECTS OF SIMULTANEOUS RADIOFREQUENCY RADIATION AND CHEMICAL EXPOSURE OF MAMMALIAN CELLS: VOLUME II

INTRODUCTION

General Information

The series of investigations summarized in this report were initiated to answer two basic questions: The first was whether or not radiofrequency radiation (RFR) in the microwave range, and specifically at 2.45 GHz (pulsed wave), at moderate power levels and specific absorption rates, was genotoxic in mammalian cell systems. The second question was whether or not the RFR would so interact with genotoxic chemicals (for example, mitomycin C, Adriamycin, and proflavin) as to increase or decrease the genotoxic activity of these chemicals. The endpoints studied included effects on cell growth, mutation at a specific locus, sister chromatid exchange induction, and chromosome aberration induction.

Dosimetry Considerations in Far Field Microwave Exposure of Mammalian Cells

In vitro investigations designed to study the effect of non-ionizing electromagnetic radiation (EM) on mammalian cells have employed a large number of exposure devices. Among such devices are: parallel plate systems (e.g., with liquid medium or air between the plates [transverse electric mode/Crawford cells]); wave guide systems (with the sample inserted in the wave guide, or end loaded); broadcast systems (from an antenna or antenna horn); microwave ovens (with the animals exposed inside, or outside with the door off); diathermy devices; and hyperthermia equipment. The early literature (and, in some instances, the current literature) on bio-electromagnetics, using these and other exposure devices, is replete with references which fail to adequately describe either the exposure system, the exposure geometry, the dosimetry of the EM radiation, or the thermal dosimetry or monitoring system.

In our laboratory (at the University of Texas Health Science Center at San Antonio), we are currently studying the possibility of an interaction between genetically hazardous chemicals and simultaneous exposure to pulsed wave RFR (microwave radiation) at 2.45 GHz. In the experiments being conducted, three factors must be monitored and/or controlled. The first is the temperature increase which occurs in the tissue culture medium in the T-25 flasks subjected to RFR at moderate power densities. The second factor is the possible non-uniformity at different positions in the RFR field. The third factor is the possible establishment of a chemical gradient in the micro-environment of individual cells, if they are allowed to remain stationary while they are being exposed simultaneously to chemicals and RFR. To alleviate these factors, a special rotating water bath exposure system has been designed and described (Meltz et al., 1988).

By use of this RFR and water bath in vitro cell culture exposure system, the impact of several positional and experimental variables on the measured specific absorption rate (SAR) for a constant power density and net forward power was examined. The variables included the effect on the SAR of: (a) the positioning of the long axis of the flask parallel or perpendicular to the long axis of the antenna horn; (b) exposing the flasks while in rotation vs. in a stationary position; (c) different volumes of medium in the flask; (d) taking temperature measurements for SAR calculation at different depths in the medium in the flasks; and (e) the presence or absence of cells while the measurements were made.

Absence of Mutagenic Interaction Between Microwaves and Mitomycin C (MMC) or Proflavin in Mammalian Cells

Evidence for possible harmful effects of RFR in the microwave range on the genetic apparatus of cells has led to comments in recent reviews (Leonard et al., 1983; Roberts et al., 1986). Some of the published reports cited in these reviews suggest a positive effect, but often lack information about the techniques used or the dosimetry (Meltz and Erwin, 1987). No determination could therefore be made as to whether the effect was due to any mechanism other than the heating of the cells.

In our laboratories, we are currently performing a series of investigations to examine the question of possible genetic effects of RFR. The endpoints being studied include effects on: DNA repair (Meltz et al., 1987a); mammalian cell mutagenesis (Meltz et al., 1987b, 1987c); sister chromatid exchange (Ciaravino et al., 1987); and chromosome aberration induction (Kerbacher et al., 1987). While theoretical considerations (Kohli et al., 1981a, 1981b) and experimental observations (Swicord and Davis, 1982, 1983) in the literature suggest a mechanism for deposition of RF energy directly in DNA, the energy of the RFR cannot be expected to, by itself, directly damage DNA. If, on the other hand, the cells were already being exposed to a genotoxic chemical at the time of RF exposure, several mechanisms in the pathway of the expression of chemical mutagenesis might be a "target," allowing for an antagonistic or additive (or synergistic) effect.

In the experiments described herein, MMC, a bifunctional alkylating agent selected because of its ability to cause DNA breaks and cross-links (Vig, 1977), was used to induce mutations in mouse leukemic L5178Y cells heterozygote at the thymidine kinase (TK) locus (Clive et al., 1973). Similar studies were performed with proflavin, which intercalates into the DNA. Forward mutation to trifluorothymidine (TFT) resistance was assessed, with and without simultaneous exposures to RFR and chemicals. The pulsed wave RFR exposures at 2.45 GHz were performed under carefully defined conditions; appropriate 37°C and temperature controls achieved by convection (water-bath) heating were simultaneously performed.

Effects of RFR and Simultaneous Exposure with Mitomycin C or Adriamycin on the Frequency of Sister Chromatid Exchanges in Chinese Hamster Ovary (CHO) Cells

The interaction of RFR and in vitro systems has been investigated, to a rather limited extent, with regard to the induction of chromosome

aberrations and sister chromatid exchanges (SCE). Yao (1982) exposed rat kangaroo RH5 and RH16 cells to 2.45 GHz radiation, and assessed the cell cultures for growth rate and chromosome aberrations. Cells which had been grown continuously in the presence of microwaves for 50 passages showed reduced growth rates, as well as an increase in chromosome aberrations. When the cells were taken out of the RFR field and observed for similar endpoints, the effects appeared reversible. Unfortunately, inadequacies in dosimetry information make it difficult to determine the nature of the RFR exposure. Alam et al. (1978) investigated the cytological effects of 2.45 GHz microwave radiation on CHO-K1 cells. These cells were exposed to 25-200 W for a 30-min period. Nuclear vacuoles, pycnotic and decondensed chromosomes were observed in cells exposed to 25-W RFR under elevated temperature conditions (49°C); this is an extremely high temperature for mammalian cells. Significant increases in chromosomal breakages per cell were also observed. When the cells were exposed to relatively high power, 75-200 W, but at hypothermic conditions (29°C), no significant increases in the same endpoints were observed. The authors attributed their results to radiation-induced temperature elevations.

Lloyd et al. (1986) exposed specimens of human blood to 2.45 GHz RFR for 20 min at SARs of 4-200 W/kg. Temperature was controlled so that it rose from 37 to 40°C. Cultured lymphocytes were examined for induced chromosomal damage, but no effect was observed.

One of the more sensitive assays for genotoxic effects is analysis of SCE induction (Latt, et al. 1981). McRee and MacNichols (1981) observed SCE frequency in bone marrow cells of mice after a 28-day exposure to 2.45 GHz RFR, 20-mW/cm^2 incident power density. The average SAR was determined to be approximately 21 W/kg. No statistically significant differences were observed in the number of SCEs between the RFR exposed and the control groups. An unresolved question is whether or not genetic damage can be induced by RFR at SARs where only small or no temperature increases result. Clearly, adequate control in dosimetry and thermometry, as well as reproducibility, are required.

Still another major biohazard consideration remains to be investigated. If the biological system is not directly susceptible to RFR-induced damage under a given set of exposure conditions, can RFR exposure cause a change in the extent of damage induced by a simultaneous exposure with a genetically hazardous chemical? In today's complex human environment, this question is equal to or more important than the effect of RFR exposure alone.

This study has been designed, using SCE induction as the biological endpoint, for the assessment of two objectives: 1) to determine if RFR alone at a frequency of 2.45 GHz, pulsed wave, power density of 49 mW/cm^2 and an SAR of 34 W/kg, can induce SCE formation in CHO cells; and 2) to determine if RFR exposure during a simultaneous treatment with MMC or Adriamycin under the same conditions can cause a change in the frequency in SCEs beyond that which is produced by the chemical alone.

Influence of RFR on Chromosome Aberrations in CHO Cells, and Its Interaction with DNA Damaging Agents

The question of whether the widespread presence of RFR in today's urban environment represents a potential public health hazard remains to be

definitively answered. Microwave radiation has been implicated in producing significant biological effects on the eye, the nervous system, the circulatory system, and the reproductive system [(for reviews, refer to: Michaelson (1971); Lin (1979); Adey (1981); and Petersen (1983)]. Relatively few studies have been carried out on the genetic consequences of RFR exposure, however; and, while effects of microwaves on inducing chromosome damage have been reported, they have largely lacked experimental detail or have not been reproduced in other laboratories (Michaelson, 1971; Petersen, 1983).

In vitro cell culture systems offer numerous advantages over in vivo systems for studying cellular responses to nonionizing electromagnetic radiation. In terms of experimental design, these advantages include the possibility of more accurate measurements of SAR and temperature monitoring, as well as facilitating the inclusion of non-RFR-induced hyperthermia controls in the experimental design. Some information is available about the relationship of hyperthermia and the induction of chromosome aberrations. Dewey et al. (1971, 1978), for example, exposed synchronous CHO cells to heat treatments of 45.5°C for varying time periods. These researchers found an increased frequency of chromosome aberrations in response to heating; this finding appeared to show cell cycle sensitivity. In contrast, Lloyd et al. (1986) reexamined the role of heating in increasing the frequency of chromosome aberrations at a more conservative temperature. Incubation of whole human blood at controlled temperatures of 40°C for 20 min failed to produce a significant increase in the frequency of chromosome aberrations in lymphocytes over that observed at 37°C. Thus, evidence already exists that the temperature achieved is critical to the observation of chromosome aberrations in mammalian cells.

An early investigation utilizing an in vitro system which appeared to implicate short-term microwave treatment in the induction of chromosome aberrations was conducted by Chen et al. (1974). This group exposed CHO cells and human amniotic cells to 2.45 GHz microwaves at intensities of 20 - 85 mW/cm^2 for varying time periods, and thus suggested an increased frequency of chromosome aberrations that appeared to be non-thermal in nature. In contrast, Alam et al. (1978) exposed CHO-K1 cells to 2.45 GHz microwaves at an incident power of 25-200 W for a 30-min period. Increased frequencies of chromosomal breakage were observed at low power (25 W) under conditions of unregulated temperature leading to hyperthermia (49°C), but not when cells were exposed to higher powers of 75 - 200 W (power density exceeding 200 mW/cm^2), with controlled temperature under hypothermic conditions (29°C). Alam et al. (1978) concluded that a radiation-induced rise in temperature was needed for the formation of chromosome aberrations. Unfortunately -- as in the Chen et al. (1974) study -- the SAR, if measured, was not reported. When Lloyd et al. (1986) exposed whole human blood to 2.45-GHz microwave radiation at 4-200 W/kg during a 20-min period, with no increase in sample temperature, no increase above control was reported in chromosome aberrations.

Yao (1982) investigated the cytogenetic consequences of chronic microwave exposure on rat kangaroo RH5 and RH16 cells. Cultures were incubated in a 2.45 GHz microwave oven converted into a microwave incubator, for 50 subculture passages; the cultures were then returned to a conventional incubator and grown for 30 additional passages. Culture

temperature in both microwave and conventional incubators was maintained at 37°C. The SAR of the medium and cell culture was tested and estimated to be 15.2±1.8 mW/g. Yao (1982) observed that the frequency of chromosome breaks increased in both irradiated cell types after 20 passages, but decreased greatly after 30 additional passages in the conventional incubator.

The objective of the present investigation was twofold: The first aim was to evaluate the ability of RFR at 2.45 GHz to induce chromosome aberrations in mammalian cells under conditions of rigorous temperature monitoring and dosimetry, as well as incorporating guidelines and recommendations for RFR research put forth by Guy (1975) and Meltz and Erwin (1987). The second aim was to determine whether simultaneous RFR exposure could modulate the action of chemicals which were known to induce DNA damage and, specifically, chromosome aberrations (Vig, 1977). The chemicals, acting by different mechanisms, could hypothetically have their initial damage, or the repair thereof, altered by the simultaneous RFR exposure, thereby altering the extent or nature (type) of the induced chromosome aberrations. This alteration, again hypothetically, might depend on the mechanism of action of the chemical. The agents used in this study were: 1) mitomycin C, which is reported to induce the formation of DNA cross-links (Iyer and Szybalski, 1963), and acts as a mono-and bi-functional alkylating agent (Vig, 1977); and 2) Adriamycin, an intercalating antibiotic (Kimler et al., 1978), which has been noted to cause single and double strand breaks in DNA (Vig, 1977). CHO cells were chosen as the mammalian cell type to be used in this investigation because, as noted by Au and Hsu (1982), this type meets most requirements for in vitro cytogenetic toxicological testing; i.e., CHO cells have good chromosome characteristics, a relatively stable karyotype, and a short generation time.

METHODS AND MATERIALS

Dosimetry Considerations in Far Field Microwave Exposure of Mammalian Cells

Water-bath Design and Construction

The exposure water bath is shown in Figure 1. Ten (10) T-25 flasks are placed in impressions cut into the underside of a Styrofoam wheel (Fig. 2); the wheel (diameter = 35.5 cm; thickness = 3.4 cm) floats freely on the surface of the water (partially buoyed by the flasks) about a hollow central Plexiglas axle of 2.5 cm outside diameter. Warm water is pumped from external adjustable temperature water baths (e.g., Brinkmann Lauda Model RC20), via plastic tubing through small inlets on the sides of a Plexiglas tank, with the water jets directed at an angle against the side of the Styrofoam wheel. This process causes the wheel to rotate at approximately 20 rpm, while simultaneously controlling the water temperature. The rotation is designed to provide averaging of the RFR field-induced absorption inhomogeneities (to be described), and also a slight agitation of the culture medium in the flasks to prevent the formation of a chemical gradient.

The water bath, constructed of Plexiglas^R, measures 45.9 x 45.9 x 11.8 cm (Fig. 1). The thickness of the Plexiglas used for the bottom and sidewalls is 1.1 cm. Four independently adjustable leveling feet, machined from Nylon^R, are attached to the base. A water inlet and an outlet port are set into each of two opposing sides of the water bath. The inlets, attached at a 45-degree angle, are constricted to generate the water jet just described. The outlets are in the same wall as the inlets, at a 90 degree angle to the wall, and are 1/2 in. lower than the inlets. A thin Plexiglas cover, 46 cm x 46 cm, with a large center circular cut-out, is placed over the water bath to reduce evaporative water loss and its concomitant cooling.

Temperature Measurement

The temperature is monitored using a BSD Medical Corp. Model 200 Thermometry Unit, to which non-interactive Vitek probes are connected. Cable extensions are used to bring the probes from the BSD unit, located outside the anechoic chamber, into the chamber. The leads then pass up through the cylindrical center axle of the water bath, and down through a pre-positioned glass capillary tube inserted in a hole drilled through the Styrofoam wheel, and then through a hole in a sham T-25 temperature monitoring flask containing medium. The probe itself is within a smaller capillary tube, with only the probe tip protruding; this tube is inserted into the larger pre-positioned capillary tube. This arrangement permits continuous temperature measurement during dosimetry experiments, and during actual RFR exposures of cells in medium in the flasks.

Exposure Facility

These experiments were performed in a 6.1 x 3.1 x 2.7 m anechoic chamber (Anechoic Chamber No. 2, in Building 1187, Radiofrequency Research Laboratory, U.S. Air Force School of Aerospace Medicine [USAFSAM], Brooks Air Force Base, TX.) with the air temperature being maintained at 37°C. The RFR was transmitted from a rectangular horn (Struthers Electronic Corp. Model 110M) in a vertically downward direction at a frequency of 2.45 GHz. The transmitter used was a Cober Electronics, Inc., Model 1831 High Power Microwave Generator. For pulsed wave exposures, a Hewlett Packard Model 8011A pulse generator was employed. The horn to water bath distance was 1.6 m; the surface of the table supporting the water bath was 53 cm above the top of the absorbing material floor of the anechoic chamber. The horn to sample distance placed the sample inside the near field, just short of the beginning of the far field. The power density in the center of the field for 600-W net forward power, at the surface of the Styrofoam table supporting the water bath, was 48.8 mW/cm^2. A Narda Probe (Model 8326B) was used for the power density measurement.

EDITOR'S NOTE: For the convenience of the reader, all illustrations and tables have been grouped at the close of this report.

SAR Determination

For determining the SAR, the liquid in the desired volume was pipetted into the T-25 flasks. For stationary exposure measurements, four probes were used simultaneously in opposing flask positions; additional monitoring probes were located in the outer heating bath, in the water in the exposure water bath, and in the air in the anechoic chamber.

Before RFR exposure, the pump-circulation-water bath system was allowed to equilibrate to 37°C. Temperature measurements at 12-sec intervals were then initiated; and, after stabilization was confirmed, the RFR was transmitted downward toward the top of the water bath. During the RFR exposure, the temperature measurements continued as before. Within 10 min of the onset of RFR exposure, when the rate of temperature increase had shown a slowing from the initial rate of increase, the transmitted power was turned off. The measurements continued until the temperature in the flask decreased to the exposure water bath temperature, which was also decreasing (Fig. 3). Heating and cooling data were collected three to five times in each position, and were entered into an SAR computer program originally designed and described by Lozano (1982), and modified by Padilla (1986) to take into account the retarding effect of the water in the exposure water bath.

Absence of Mutagenic Interaction Between Microwaves and Mitomycin C or Proflavin in Mammalian Cells

RFR Exposure Parameters

The horn to water bath distance was 1.6 m, thus placing the sample inside the near field just short of the far field. The average net forward power in the mitomycin experiments was 500 W. The pulse width was 10 μsec, and the pulse repetition rate was 25,000 pps; the duty factor was 0.25. The power density at the table surface used to support the water bath was 48.8 mW/cm^2 (600 W forward power); the power density at 500 W was 40.7 mW/cm^2. The SAR was 30 W/kg. Power density measurements were made using a Narda Model No. 8616 power meter with a Narda Model No. 8644 Probe. In Proflavin experiments 1 and 2, the forward power was 500 W; the power density was 87.5 mW/cm^2, with an SAR of 40.8±13.4 W/kg. The maximum temperature achieved was 39.5°. In Proflavin experiment 3, the forward power was 600 W; the power density was 64.6 mW/cm^2, with an SAR of 40 W/kg. The maximum temperature achieved was 38.8°C. An AEL Model 5001 antenna horn was used for this exposure. The temperature was monitored continuously before and during the 4-hr RFR exposure in the chamber air, in a flask in each of the three water baths, and in the water in the water baths, using a BSD Model 200 thermometry unit with non-RFR interactive Vitek Probes. In each experiment, up to 10 flasks were placed into cut-outs on the underside of a Styrofoam wheel, which was free to rotate on the water surface inside each of the water baths. By means of jets of water (from the circulating water system) impinging on its side, the Styrofoam wheel was

kept in constant circular motion; this served to insure homogeneity of RFR exposure and some mixing of the chemical with the cells.

Mutation Assay Procedure

The cell line employed was the L5178Y mouse leukemia heterozygote obtained from Dr. Donald Clive (Genetic Toxicology Lab., Burroughs-Wellcome Corp., Research Triangle Park, N.C.). The procedures used for the assay are similar to those previously used in our laboratory (Meltz and MacGregor, 1981). Before each experiment, the cell stock was purified of spontaneous mutants lacking thymidine kinase activity, by using a standard 24-hr THMG* treatment. The cells were then seeded at a low cell density for incubation over the weekend in recovery medium (THG medium)**, and diluted (when necessary) on the following Monday prior to transport from the tissue culture laboratory at the University of Texas Health Science Center at San Antonio (UTHSCSA) to the tissue culture laboratory at USAFSAM. The flasks were placed in a New Brunswick Rotating Incubator for overnight incubation at 37°C. On the following day, the cell density of the mass culture was determined by Coulter Counter (Model ZBI, Coulter Electronics, Hialeah, Fla).

An appropriate volume with the desired number of cells was then transferred into 50-ml sterile centrifuge tubes (Corning) and centrifuged at 750 rpm, at room temperature, in a Damen/IEC Division Clini-cool table-top centrifuge. An appropriate volume of the residual F_{10p} medium was left in each tube so that, after resuspension by pipet and transfer into a 250-ml flask with warm (37°C) Fo (Clive et al., 1979), the combined volumes of F_{10p} and Fo resulted in a final serum concentration of 3% (F_{3p}) and a cell density of 600,000 cells/ml. This suspension was then rotated for 15 min at 37°C, at which time 7.92-ml aliquots were distributed into 24 plastic T-25 flasks (Corning). Each flask was gassed for 1 sec with a mixture of 5% CO_2 and 95% air, and the caps were tightly sealed. The flasks were then transported, in an insulated chest (just large enough to hold the 24 flasks), from the tissue culture laboratory to the RFR exposure facility (a travel time of 15 min). On arrival, the flasks were either temporarily placed in a 37°C incubator, or immediately placed under a Lab-Tek table-top hood for the addition of 80μL aliquots of either F_o, MMC in F_o or proflavin in F_o, or filter-sterilized ethyl methane sulfonate (EMS) (dissolved in saline). The initial EMS solution was a 100 X concentrate of the desired final concentrations of 0.1, 0.2, or 0.3 μg/ml MMC, 1 μg/ml proflavin, or 1.0 mg/ml EMS.

The mutagenic chemical and F_o solvent control aliquots were not added to the flasks until theespecially designed water baths and warm-water circulating systems had stabilized at 37°C, and the air temperature in the

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* THMG: Fisher's Medium for Leukemic Cells of Mice (Grand Island Biological Co., Grand Island, N.Y.) (F_{10p}; Clive et al., 1979) containing 3.1 x 10^{-5}M thymidine, 9.2 x 10^{-5}M hypoxanthine, 2.2 x 10^{-5} μg/ml methotrexate, and 2.5 x 10^{-4}M glycine.

**THG: Same as THMG, but lacking the methotrexate.

anechoic chamber was constant at 37°C±0.2°C. Six different conditions were examined in each experiment: 4 flasks were exposed only to RFR; 4 flasks were exposed simultaneously to RFR and chemical; 4 flasks were exposed simultaneously to chemical and temperature control (TC) conditions; 4 flasks were maintained as a 37°C control; 4 flasks were treated with chemical at 37°C ; 2 flasks were incubated under TC conditions, but without chemical. In addition, 2 flasks were treated with EMS at 37°C. Immediately after addition of chemicals, the flasks were placed into positions in the underside of the circular Styrofoam float (Fig. 2) (10 per float) in the appropriate (one of the three) water baths (RFR, TC, or 37°C) in the anechoic chamber. The water bath for the RFR exposure was at one end of the anechoic chamber. The two other baths (37°C and TC) were at the other end of the anechoic chamber, out of range of the RFR.

Circulating water pumps outside of the anechoic chamber were used to establish the initial 37°C temperature in all three water baths. When all of the flasks were in place, the temperature-control water-bath pump was turned up above 37°C. As the temperature in the TC monitoring flask (in the Styrofoam wheel) began increasing, 5 - 6 min later, the RFR exposure at 500-W net forward power was started. The TC water-bath heater was manually adjusted so that the continuously monitored temperature in the TC flask medium closely followed that in the RFR exposure flasks (Fig. 4).

The RFR exposures continued for 4 hr; the 37°C and TC flasks were incubated for the same time. A rapid rise in medium temperature (Fig. 4) over the first 20 min was followed by a slow approach to RFR-induced maximum temperatures at 4 hr, ranging from 38.9 to 39.9°C in five different experiments. No cooling was applied to the RFR-exposed water-bath water supply; the controller was left at the initial 37°C setting.

Immediately after the RFR exposure, the flasks were transported back to the tissue culture laboratory in the Styrofoam chest. The contents of each flask were separately transferred by pipet into sterile 15-ml conical (Corning) centrifuge tubes, and spun at room temperature for 8 min at 750 rpm in the table-top centrifuge. The treatment supernatant was removed from each tube by aspiration, and 10 ml of fresh warm medium was added. The cells in each tube were then resuspended by pipet. Two additional washings by centrifugation were performed, with the cells finally being resuspended in 10 ml of F_{10p}. From each tube, either a 4-ml or 5-ml aliquot was transferred into an autoclaved (and silicon-rubber stoppered) 50-ml glass Erlenmeyer flask already containing 6 ml or 5 ml of warm F_{10p}. The flask had been pre-gassed for 1 min with a mixture of 5% CO_2 and 95% air. These flasks were then incubated in the dark at 37°C in the rotating incubator for an expression period of 60-70 hr. Daily cell density determinations and dilutions each day to 300,000 cells/ml were performed. The remainder of the mutation assay, using 1 μg/ml of TFT for selection, has been described (Meltz and MacGregor, 1981). Three viability dishes and three mutation assay dishes were prepared for each of the treatment flasks. The colonies in 0.4% BBL agar were counted electronically after 11 days of incubation at 37°C; an Artek Colony Counter (Model 880; Artek Systems Corp., Farmingdale, N.Y.) was used. Individual dish viabilities were determined and averaged; this average was used to calculate individual dish mutation frequencies, which were subsequently averaged to give one value for each initial treatment flask (2 or 4 replicates).

Effects on Cell Growth

On the day of exposure, after the transfer of the initial volume of cells to the expression flasks, the cell density of each suspension was determined by Coulter count. A volume of each suspension containing 1.2 x 10^6 cells, was pipetted into a Corning 250-ml, plastic, screw-capped Erlenmeyer flask containing 60 ml of F_{10p} (pre-gassed as already described). The resulting initial cell density was approximately 20,000 cells/ml. These flasks were incubated at 37°C in the New Brunswick rotary incubator. Cell densities were determined immediately, and then daily, by sampling two 0.5-ml aliquots from each of two growth flasks per initial treatment flask. This procedure, not usually performed in L5178Y mutation studies, permitted a very accurate examination of the effect of the different treatment conditions on L5178Y cell growth.

Effects of RFR and Simultaneous Exposure with Mitomycin C or Adriamycin on the Frequency of Sister Chromatid Exchanges in CHO Cells

Cell Line

Chinese hamster ovary cells were maintained, in T-25 flasks (Corning Glass Works, N.Y.), in Ham's F-10 medium supplemented with 10% fetal calf serum and gentamycin (GIBCO, Grand Island, N.Y.) (25 μg/ml). Stock cultures were kept at 37°C in a 95% air and 5% CO_2 environment.

Preliminary MMC Experiments

Preliminary experiments were conducted to determine the extent of SCE induction in CHO cells under various conditions of MMC (SIGMA, St. Louis, Mo.) exposure. At 24 hr before MMC treatment, stock cultures of cells were resuspended, using 0.025% trypsin, and distributed in 8 ml volumes into T-25 flasks at 2 X 10^5 cells per flask. Triplicate flasks of cells were used for each of the treatment conditions. After each exposure the cells were rinsed with fresh medium and incubated at 37°C in the presence of Bromodeoxyuridine (BrdUrd) for 24 hr. The protocol for SCE analysis is described later in this section.

RFR Experiments

At 24 hr before the RFR exposure, stock CHO cultures of cells were resuspended, using 0.025% trypsin (GIBCO), and distributed in 8-ml volumes of medium into T-25 flasks at 2x10^5 cells/flask. These cells were incubated for 24 hr at 37°C to permit attachment and proliferative growth. For each experiment, 18 flasks of cells were required; triplicate flasks were used for 6 treatment conditions. The treatment conditions included: 1) 37°C control; 2) MMC or Adriamycin treatment at 37°C; 3) temperature control (TC); 4) MMC or Adriamycin treatment with TC; 5) RFR exposed; and 6) MMC or Adriamycin treatment with RFR exposure. During RFR exposure, a rapid rise occurs in the temperature of the medium within the exposed flasks. A TC condition was therefore included in these experiments, and was accomplished by increasing the temperature in a water bath to follow closely the temperature increase in the medium of the RFR-exposed flask. All three water baths (RFR, TC, and 37°C) were positioned within the (6.1 x 3.1 x

2.7 m) anechoic chamber; the air temperature was regulated at 37°C. The TC and 37°C water baths were at the far end of the anechoic chamber, isolated from the cells being exposed to the RFR.

The physical exposure parameters of the experiments are given in Table 1. The RFR was transmitted from a rectangular horn (Struther Electronic Corp., Model 110M) in a vertical downward direction. The transmitter was a Cober Electronics, Inc., Model 1851 High Power Microwave Generator. Power densities were determined at the surface of the Styrofoam exposure table at nine points within the dimensions of the water bath. A Narda Probe (Model 8326B) was used. In the center of the field, measurements were taken with the probe oriented in nine directions. These values were averaged, and then corrected by the probe specific factor. The resulting value was used to correct the unidirectional measurements at the nine points initially measured. The variation about the mean ranged up to 17%. The SAR of culture medium in the T-25 culture flasks positioned beneath the Styrofoam float in the water bath was determined using a procedure previously described (Lozano 1982), and recently modified (Padilla, 1986). A BSD Medical Corp. (Salt Lake City, U.) Model 200 Thermometry unit, with non-interactive Vitek leads, was used to make the temperature measurements.

Immediately before the RFR exposure, concentrated MMC was added to the appropriate flasks to give a final concentration of 1×10^{-8}M; the final Adriamycin (ADR) concentrations were 0.45 or 0.58 μg/ml. All flasks contained 8 ml of medium. The flasks were then loaded into the bottom of a Styrofoam wheel which could rotate freely about the central axis of a Plexiglas tank. Warm water circulated from external water baths (one for each exposure condition) was pumped through small inlets on the sides of the Plexiglas tanks and directed at the side of the Styrofoam wheel, causing the wheel to rotate at a speed of approximately 20 revolutions per minute (rpm). This speed enabled the exposure of the flasks to be homogeneous with respect to the RFR field, and also permitted some mixing of the chemical in the medium within the flasks.

All the water baths were adjusted to 37°C before the start of the experiment. Approximately 5 min before the initiation of RFR exposure, the thermostat on the TC water bath was turned up so that the TC and RFR temperature profiles would be similar, once the field was turned on. The RFR exposure period was 2 hr.

The temperatures in all three water baths were monitored continuously for a period preceding the onset of RFR exposures, as well as during the 2-hr exposure period. A representative temperature profile is shown in Figure 5. The importance of the temperature profile is twofold: 1) the rates at which the temperatures of TC and RF samples rose was kept approximately similar; and 2) when the RF sample reached equilibrium, the temperature of the TC control was also stabilized for the remainder of the exposure.

After exposure, the medium in all of the flasks was removed and each flask was rinsed with fresh medium. The flasks were then filled with 8 ml of fresh medium containing 10μM BrdUrd (Sigma, St. Louis, Mo.) and incubated at 37°C in the dark for 24 hr. At 21-hr post-treatment, 0.2 ml of colcemid (GIBCO) (10μg/ml) was added to the cultures. Three hours later, gentle

shaking was used to detach mitotic cells, and the medium containing these cells was removed. Slides of the mitotic cell suspensions were then prepared and stained by the fluorescence plus Geimsa (FPG) technique of Perry and Wolff (1974). The slides were coded, and the frequency of SCEs for each slide was scored. Fifty cells were scored for each treatment group. The experiment was conducted four separate times. The data were analyzed with a two-way analysis of variance (ANOVA) test. The ANOVA table combined the results of the 4 experiments, and determined significance of experimental error as well as treatment error.

Influence of RFR on Chromosome Aberrations in CHO Cells, and Its Interaction with DNA Damaging Agents

Cell Line

Chinese hamster ovary cells were used exclusively in these studies. The cells were grown in 75 cm^2 flasks at 37°C in 25 ml of Ham's F-10 (Grand Island Biological Co. [GIBCO], Grand Island, NY); supplemented with 10% fetal bovine serum (GIBCO) and 0.8% penicillin/streptomycin (GIBCO; 10,000 U/ml penicillin and 10,000 µg/ml streptomycin) ("complete Ham's F-10"). The cells were maintained under an atmosphere containing 5% CO_2 and 95% air.

RFR Experiments

Radiofrequency exposures were conducted at the USAFSAM Radiofrequency Research Facility. Experiments were conducted in a 6.1 x 3.1 x 2.7 m anechoic chamber with the air temperature thermostatically regulated to 37°C ± 1°C. RFR was generated by using a Cober Electronics, Inc., Model 1831 High Power Microwave Generator, and transmitted in a vertically downward direction from a rectangular horn (Struthers Electronics Corp. Model 110M). The horn to experimental flask distance was 1.6 m; the cells were exposed in the near field just before the beginning of the far field. The T-25 flasks containing the cells to be RFR-exposed were placed into impressions on the underside of a Styrofoam disk having positions for 10 flasks. The disk was rotated during RFR exposure by jets of warm water impinging on its side at an angle. (The water-bath exposure system has already been described). The physical parameters for the microwave exposures were as listed in Table 1, and yielded, under these conditions, an SAR of 33.8 W/kg when a volume of 8 ml of complete Ham's F-10 was over the cells in the flasks. The SAR was determined by using a procedure previously described by Lozano (1982), as modified by Padilla (1986).

Two other water baths of identical design were at the other end of the chamber, outside the limit of measurable RFR. These baths were used either for the TC incubations or the 37°C incubations, which were performed simultaneously with the RFR-exposed samples. The 10 positions under the disk in each bath were filled either with flasks containing CHO cell monolayers or with 8 ml of water; one flask contained 8 ml of Ham's F-10 for continuous temperature monitoring. This monitoring was performed by using a BSD Medical Corp. Model 200 Thermometry unit equipped with non-interactive Vitek probes. Besides continuous measurement of medium temperature in one of the flasks in each water bath, chamber air temperature and bath temperatures were also monitored during the 2-hr exposure period.

All experiments were conducted using an in situ culture methodology in which cells were grown on coverslips (Schmid, 1975). Twenty-four hours before the experiment, T-25 tissue culture flasks were labeled and a sterile coverslip (14 x 54 mm) was inserted into each flask. After being washed with 10 ml of Ham's Balanced Salt Solution (HBSS without Ca^{+2} and Mg^{+2}) at 37°C, CHO cells were detached using trypsin (0.25%; KC Biological Co., Lenexa, Kan.) from a $75cm^2$ T-flask. The cell density was determined using a hemocytometer. The flasks containing the sterile coverslips were seeded with 1.2×10^5 cells in 8 ml of fresh complete Ham's F-10, gassed with 5% CO_2 and 95% air, and incubated overnight at 37°C.

On the day of the experiment, the chamber air temperature and the temperature of the water in the three water baths inside the anechoic chamber were adjusted to 37°C. When all of the baths had been equilibrated, 80 μL of a 100X concentrate of the test chemical, MMC or ADR dissolved in complete Ham's F-10, were added to the appropriate flasks. The final MMC concentrations used in the RFR exposures were 0.075 and 0.1 μg/ml; the ADR concentration used was 0.175 μg/ml. An 80-μL volume of Ham's F-10 was used in non-chemical exposed flasks. The sample flasks, the sham flasks, and the temperature monitoring flasks were then inserted into the circular Styrofoam holders, which were lowered into the appropriate water bath. When the temperature of all flasks had equilibrated to 37°C (5 - 10 min), the temperature of the TC bath was increased. After 4 - 6 min, as the temperature in the TC flask began to increase, the RFR exposure was begun. The timing was such that the temperature rise in the medium of the TC flask closely followed in time the temperature rise in the medium of the flask exposed to RFR (e.g., Fig. 5). Upon completion of the 2-hr RFR exposure, the flasks were removed and the medium was pipetted from each flask and discarded. The total chemical exposure time was 2-1/2 hr. The flasks were washed thoroughly twice with 10 ml of HBSS (with Ca^{+2} and Mg^{+2}) to ensure removal of trace amounts of chemical. Complete Ham's F-10 (8 ml at 37°C) was then pipetted into each flask, and 15 μl of colcemid added (0.02 μg/ml final; GIBCO). The flasks were incubated for 18 hr at 37°C in an atmosphere of 5% CO_2 and 95% air.

The culture medium was removed and 10 ml of hypotonic solution (0.075 M KCl) was carefully added to each flask designated for chromosome analysis; these flasks were incubated at 37°C for 15 min. All subsequent steps were carried out at room temperature. Two ml of fixative (3:1 methanol:acetic acid) were then added to each flask. After 2 - 3 min, the hypotonic solution plus fixative was carefully removed by pipette. Fresh fixative (10 ml) was added for 2 - 3 min, and then removed. Ten (10) ml of fresh fixative was then added for 2 hr. With the fixative still present, the flasks were cut open and the coverslips removed. In some cases, gentle steaming for approximately 3 sec was employed to enhance spreading of chromosomes. The coverslips were affixed to clean glass microscope slides. After air drying for 24 hr, the slides were stained for 3 min in 4% giemsa (Fisher) in Sorensen's buffer. Prior to analysis, slides prepared from untreated and chemically treated cultures were blinded by covering the labeled area with opaque tape, and were subsequently randomized.

The blinded, stained slides were first examined for chromosome aberration analysis under low power (25%). Metaphase spreads were considered non-scorable if there was: poor chromosome spread; too much spread; too much variation from a circular configuration of the spread;

extensive overlapping of chromosomes; poor staining; or the presence of tetraploidy or polyploidy. The number of chromosomes within a particular spread was not a criterion for analysis, since a range in the number of chromosomes in untreated cells is inherent, and since chemical treatment can further affect the chromosome number per cell. However, with the experimental protocol employed, greater than 95% of the mitotic figures analyzed contained between 20 and 23 chromosomes. When a particular metaphase spread had been selected for analysis, it was examined by using oil immersion under high power (65X or 100X). The number of chromosomes in the spread were counted and recorded. Each chromosome in the spread was then carefully examined for chromosome aberrations by using the criteria described by Brusick (1980). Aberrations scored included: chromatid gaps (tg); chromatid breaks (tb); chromosome gaps (sg); chromosome breaks (sb); fragments (f); complete rearrangements (cr); minutes (min); terminal chromatid deletions (td); rings (r); and dicentrics (d). Specific aberration types were noted on the score sheet; if no aberrations were found, the spread was noted as "normal". Finally, the position of the spread on the slide was recorded for future referral. In this manner, 100 mitotic figures were routinely analyzed per slide. After all slides had been scored, they were unmasked; chromosome number and status of the metaphase spreads (normal vs. aberrant) were tallied. Aberration data for each condition were summarized as: percentage of aberrant cells; aberrations per cell; aberrations per aberrant cell; aberration types (number of aberrations found per 100 cells); and aberration events (number of aberrations found per 100 cells). Complex rearrangements, acentric rings and dicentrics, were scored as resulting from two separate aberration events; all other aberration types were scored as resulting from one aberration event.

In this work, aberration types are distinguished from aberration events. The basis for this distinction lies in the fact that a number of chromosome aberration types (namely, complex rearrangements, rings, and dicentrics) are believed to arise from two separate aberration events. Thus, cr, r, and d are each scored as two aberration <u>events</u>, but as only one specific aberration <u>type</u>. This distinction was used since it was not known whether a particular type of aberration would be preferentially formed as a consequence of an exposure condition.

Single flasks chosen randomly from each exposure condition were processed for mitotic index analysis, using standard techniques and excluding hypotonic treatment. Cells removed by trypsinization and suspended in original medium were fixed three times with methanol:acetic acid prior to dropping on clean microscope slides. After drying, slides were stained for 3 min with 4% giemsa. Blinded slides were analyzed for percent mitotic cells. After analysis, the slides were unmasked and the mean mitotic index$\pm$the standard error of the mean (S.E.M.) was calculated for the replicate slides in each condition.

Preparation of slides for determining the extent of escape of CHO cells from colcemid-induced metaphase arrest was identical to that of slides for chromosome aberration analysis, except that the 18-hr incubation was carried out in the presence of 10-μM bromodeoxyuridine (BrdUrd) in addition to colcemid. Coverslips mounted on slides were stained by the fluorescence-plus-giemsa (FPG) method of Perry and Wolff (1974). Blinded slides were analyzed by counting scorable cells as first or second mitotic, based on the

absence or presence of differential chromatid staining (Perry and Wolff, 1974). No third division cells were detected under these conditions.

The data, as presented herein, comprised pooled results from experiments ranging in number from two to seven. Within a particular experiment, each exposure condition (i.e., 37°C Control ± chemical; Temperature Control (TC) ± chemical; and RFR Treated ± chemical) was composed of three separate cultures, from each of which one slide was prepared. One hundred (100) chromosome spreads, chosen based on criteria already discussed in detail, were carefully examined for the presence of chromosome aberrations; these were then tallied. Aberration type data were also summarized in terms of total aberrations found, allowing for a perhaps more meaningful comparison of levels of chromosome aberrations in general in the various exposure conditions. Results of a given experiment thus consisted of three individual data points for specific aberration types, allowing for calculation of (S.E.M.) as well as single-tailed analysis of variance. Pooled data as presented were not arrived at by manipulating mean ± S.E.M. for individual experiments; rather data from individual slides were analyzed, and statistical comparisons made between the various exposure conditions in each experiment, as well as between experiments by two-tailed analysis of variance.

In the tabular presentation of the data obtained in these experiments, only differences showing a 95% level of confidence are considered to be statistically significant. All data, in the summary tables, which have not been specifically indicated by an asterisk did not meet this criterion.

RESULTS

Dosimetry Considerations in Far Field Microwave Exposure of Mammalian Cells

Effect of Positioning the Long Axis of the T-25 Flask Perpendicular or Parallel to the Long Axis of the Antenna Horn

The specific absorption rate data in Table 2 are the result of three replicate experiments performed on different days. The measurements in two parallel or two perpendicular positions were performed simultaneously. The experiments were performed using volumes of medium (F_{10p}) [Fischer's Medium for leukemic cells of mice, GIBCO (Grand Island, N.Y.) with 10% heat inactivated horse serum and other tissue culture additives] of either 8 ml or 12 ml.

As can be seen in Table 2, the average values (at a given volume) were always similar for opposing flask positions (on opposite sides of the axle), pointing toward the field center and simultaneously having their long axes parallel to or perpendicular to the horn long axis. The positioning of the T-25 flasks with their long axes parallel to the long axis of the antenna horn resulted in a higher SAR than when their long axes were perpendicular to the axis of the antenna horn. In both cases, the larger volume of medium resulted in the measurement of a lower SAR (discussed in a later section).

Effect of Flask Rotation During RFR Exposure on the Measured SAR

These data are summarized in Table 3, for two different medium (F_{10p}) volumes. In these experiments, performed with rotation, only one probe at a time can be used for temperature measurement. Because of the continuous rotation of the flask, the probe is subject to slight rises and falls in the medium in the flask as the probe lead rotates in its glass capillary sheath. Even with this small perturbation, the measurement variability was very small. At both volumes, the measured SAR values are intermediate to those measured with the flasks stationary in the perpendicular and parallel positions.

Effect of Medium Volume

The effect of medium volume in the flasks on the measured SAR was then determined at 2.45 GHz. The measurements were made simultaneously in four T-25 flasks positioned in the stationary Styrofoam holder beneath the corners of the rectangular antenna horn. The flask caps were pointed toward the center axle.

The SAR data reported in Table 4 are the result of three to five different runs performed on different days. The average SAR±S.D. (standard deviation) in W/kg is given for each position measured with each volume. When the means of all positions were determined, the values were 36.5, 33.8, 31.3, 26.1, and 23.9 W/kg for the volumes 8,9,10,11 and 12 ml, respectively. For any of the five volumes tested, the average value of any one flask position varied from the mean of all positions at that volume by no more than 13.1%. There was clearly a decrease in the measured SAR with increasing medium volume.

This volume effect was further explored by pipetting 40 ml of medium into a T-25 flask, and measuring the temperature (for SAR calculation) at different heights in the medium in the flask. As indicated in Figure 5, a steady increase was noted in the calculated SAR as the surface of the medium (closest to the antenna horn) was approached. These observations emphasize the care which must be used in determining the SAR for dosimetry purposes in an in vitro system.

Effect of Presence of Cells in the Medium in Which the SAR is Measured

In these experiments (summarized in Table 6), SAR measurements were made in F_{3p} medium (with 3% horse serum), with L5178Y mouse leukemia cells in suspension. The flasks were in rotation during the measurements. Twelve ml of medium were present in the T-25 flasks. At a cell density greater than 500,000 cells/ml, the mean measured SAR with cells present was similar to that without; i.e., 15.5±0.9 W/kg (with cells) vs. 15.4±0.6 W/kg (without cells).

Similar experiments were conducted (Table 7) for surface-attached CHO cells. The medium was BME [BME medium is Basal Medium Eagle (BME) (with Earle's salts] supplemented with 10% fetal calf serum. The flasks were in rotation during the measurements. Again, using a relatively high density monolayer of cells under 10 ml of medium, no difference in SAR was observed.

The values were 26.7±1.7 W/kg, (with cells) vs. 26.2±1.6 W/kg (without cells).

Absence of Mutagenic Interaction Between Microwaves and Mitomycin C or Proflavin in Mammalian Cells

Effects on Cell Growth

For these experiments, the standard L5178Y TK±mutation assay protocol was expanded to allow for a careful investigation of any effect of microwave exposure, with or without simultaneous treatment with MMC, on the growth of L5178Y cells in suspension.

In each of these experiments, two independent flasks were separately treated for each exposure condition, and a separate growth flask (60 ml) was initiated for each independent treatment flask. In Figure 6 is shown the growth inhibitory effect of a 4-hr treatment with MMC (alone) at a concentration of 0.3 μg/ml and a constant temperature of 37°C. Also shown is the growth of the 37°C control and the 1 mg/ml ethylmethane sulfonate (EMS)-treated positive control (at 37°C). In Figure 6, each line is the average of the cell count in the two independent treatment growth flasks for each exposure condition in each of the two separate experiments.

In Figure 7 are presented the results of the two experiments in which cells were exposed to MMC under three conditions: 37°C, RFR exposed, and temperature control. The results of the two experiments are shown separately. The RFR exposure simultaneously with MMC treatment, as compared with the MMC treatment for 4-hr at 37°C, clearly did not alter the growth inhibitory activity of MMC in either an additive or antagonistic way. The maximum temperatures achieved in these two experiments were 39.1°C (Experiment 1) and 38.9°C (Experiment 2). Similarly, no effect was observed for the temperature control treatment with MMC at the same temperatures as achieved by the RFR exposure.

Effects on MMC or Proflavin Mutation Induction

In performing these experiments, 2 or 4 independent flasks were treated for each treatment condition. A growth flask (10 ml) was initiated for each treatment flask for expression; then, 3 agar dishes were prepared for viability, and 3 for TFT selection for each independent treatment flask. After cloning efficiency values were averaged for the 3 viability flasks, the mutation frequency of each of the 3 selection dishes was calculated and averaged.

In each of the experiments, the cloning efficiency and the relative survival of the cells from each treatment flask were determined at the end of the expression period. The mean percent survival values, in those experiments involving treatments at 0.3 μg/ml MMC, ranged from 6.0 to 28.1% in two experiments (six-exposure situations); in the experiments involving treatments at 0.2 μg/ml MMC, these values ranged from 6.0 to 15.5% in two experiments (six-exposure situations); and, in the experiment involving treatment at 0.1 μg/ml, the values ranged from 33.9 to 35.9% (three-exposure situations). In the three experiments performed using proflavin at 1 μg/ml, the percent survival ranged from 56.4% to 88.5% (nine exposure situations).

The individual spontaneous mutation frequencies of each of the replicate independent treatment flasks for the 37°C solvent control (in each experiment) are listed in the lower part of Table 8 (for MMC experiments) and Table 10 (for proflavin experiments). The averages which are indicated, ranging from 37.7 to 100.3 in the 5 MMC experiments, and from 72.6 to 104.2 in the three proflavin experiments reported, were then used to calculate the induced mutation frequency for each independent treatment flask. The individual replicate treatment flask mutation frequencies for the RFR-only exposure (no chemical present) are presented in the upper part of Tables 8 and 10; the induced mutation frequencies for RFR-only are presented in the upper part of Tables 9 and 11. Clearly, there is no evidence of induction of mutation in the L5178Y mouse leukemic cell line at the thymidine kinase locus by RFR under these conditions of exposure. The same is true for the TC exposures.

The results of five different mutation experiments, involving simultaneous exposure to RFR and three different concentrations of MMC, are summarized in Table 12. The individual replicate mutation frequency values for the experiments are in Tables 13 to 15. Each value reported is the average value (with standard error) for all of the replicates (independent treatment flasks) performed for each treatment condition. The results of three different mutation experiments, involving simultaneous exposure to RFR and one concentration of proflavin, are summarized in Table 16. The individual replicate induced mutation frequency values for the experiments are listed in Tables 17 to 19.

The individual values for the independent treatment flasks were subjected to both a two-tailed t-test and a two-way analysis of variance to determine if any significant differences existed between the induced mutation frequencies with MMC present under the conditions: of RFR exposure; of temperature control by convection (where the temperature was adjusted so that the medium temperature in the flasks closely followed that in the RFR exposed flasks); and at 37°C. At the 0.05% probability level, no statistically significant difference could be detected.

Effects on Colony Size Distribution

In the three experiments involving simultaneous RFR and proflavin exposure, reproducible colony size distributions were obtained for the TFT resistant (mutant) colonies. Representative size distribution profiles are presented in Figures 8 to 12. Comparison of the proflavin size distribution at 37°C (Fig. 10) to the EMS size distribution (Fig. 9), and to that of the spontaneous mutant colonies in the 37°C control (Fig. 8), shows that a bimodal distribution of the colony size occurs after proflavin treatment. When the colony size distributions after simultaneous treatment with RFR and proflavin (Fig. 11) and TC with proflavin (Fig. 12) are compared with that for proflavin treatment at 37°C (Fig. 10), no alteration of the colony size distribution is observed.

Effects of RFR and Simultaneous Exposure with Mitomycin C or Adriamycin on the Frequency of Sister Chromatid Exchanges in CHO Cells

Preliminary MMC Experiments

Summarized in Table 20 are the results of the preliminary experiments to determine the extent of SCE induction upon treatment of CHO cells with MMC under various conditions. Sister chromatid exchange frequency with respect to MMC concentration and time of exposure were determined. The objective of these experiments was to ascertain a suitable combination of concentration of MMC and exposure duration which would result in an approximate doubling of SCE frequency over control values. The combination of 2-hr exposures and MMC concentration of 1×10^{-8}M was chosen as the chemical exposure for the RFR experiments subsequently performed.

RFR Experiments

The results of four separate RFR experiments are summarized in Table 21. Each experiment was performed on a different day with a different cell population. Statistical comparisons were conducted using a two-way ANOVA in which the significance of treatment and experimental error was determined. Two-way t-tests were also conducted for evaluation of the results. No statistically significant differences were observed.

The frequency of SCE induction is presented as SCE per cell, plus/minus the standard error of the mean (S.E.M.), for each of the four experiments. Similar experiments were performed with Adriamycin at two different concentrations. Again, no statistically significant differences were observed (Table 22).

Influence of RFR on Chromosome Aberrations in CHO Cells, and Its Interaction with DNA Damaging Agents

Baseline Studies

The baseline frequency of specific types of aberrations and aberration summary observed in untreated CHO cells maintained at 37°C is shown in Table 23, and represents data from seven separate experiments. The most common abnormality encountered in these cells was chromosome gaps, followed in decreasing order by minutes, chromatid gaps, fragments, chromosome breaks, chromatid breaks, and rings. In 2100 cells examined, no complex rearrangements, terminal chromatid deletions, or dicentrics were observed. Approximately 10 aberrations were found per 100 cells analyzed. Since, in some cases, more than one abnormality was observed in a particular cell, the aberrations per aberrant cell were greater than unity.

RFR Experiments

Exposure of CHO cells to RFR alone for 2 hr in seven experiments (Table 23) resulted in tissue culture medium temperatures ranging from 38.6°C to 40.2°C. Under these conditions, no statistically significant

change, in the frequency of any of the aberration types scored or aberration summary data was observed relative to the 37°C control data. A similar lack of effect was noted (Table 23: TC) in cells which were exposed to water-bath temperatures which closely followed those elicited by RFR exposure.

Initial studies to determine the influence of simultaneous RFR and MMC exposure on the frequency of chromosome aberrations were conducted using a concentration of MMC of 0.075 μg/ml. In two experiments (Table 24), the maximum temperature achieved in RFR-exposed and TC flasks was approximately 39.5°C; under these conditions, with obvious induction of chromosome aberrations by MMC, no change in the frequency of specific aberration types relative to the 37°C control condition was observed in the cells exposed simultaneously to the chemical and RFR. Likewise, no change in the frequency of specific types, as compared with that caused by the chemical at 37°C, was observed at the increased medium temperature produced by water bath convection. Furthermore, no significant changes in the aberration summary data shown in Table 24 resulted from simultaneous chemical and RFR exposure, as compared with chemical exposure at 37°C and chemical exposure at TC conditions. Studies were also conducted using 0.1 μg/ml MMC; these data are shown in Table 25. In these experiments, the maximum temperatures achieved in RFR exposure flasks in three separate experiments averaged 38.9°C. Treatment with MMC at this higher concentration resulted in a tenfold increase in the number of aberration events observed above non-chemically treated cells (compare Tables 23 and 25). Again, as for the case of treatment with 0.075 μg/ml MMC, neither simultaneous RFR exposure nor incubation at increased temperature produced by water-bath hyperthermia resulted in statistically significant changes from the frequency of chromosome aberrations resulting from incubation with 0.1 μg/ml MMC at 37°C.

The influence of RFR exposure on the action of ADR was similarly studied. The effect of ADR (0.175 μg/ml) on the frequency of chromosome aberrations in CHO cells at 37°C is shown in Table 26. Adriamycin was shown to be less effective (on a microgram/milliliter concentration basis) than MMC in producing chromosome aberrations. In addition, the frequency of specific aberration types induced by the two chemicals appears to differ (compare Tables 24 to 26). In four experiments utilizing ADR, RFR exposures yielded a maximum medium temperature of 40°C. Analogous to the findings with MMC, simultaneous exposure to ADR and RFR, or incubation at increased water-bath temperatures and ADR, failed to significantly alter the distribution of specific aberration types from that found by ADR treatment at 37°C. Unlike the situation with MMC, however, a small but statistically significant (at 95% confidence level) increase in the total number of aberration events (and, consequently, aberrations per cell) was observed in the RFR exposed and TC conditions. No statistical difference was found however, between the aberrations per cell induced by ADR and RFR, and that from ADR incubated under TC conditions.

The mitotic index of CHO cells was also determined for each of the various conditions in the experiments just described. In seven experiments, cultures maintained at 37°C were found to have a mitotic index of 7.9 ± 0.8. Neither RFR exposure nor increased water-bath temperatures had a significant effect on this value. While treatment with 0.075 μg/ml MMC also did not result in a significant alteration of the mitotic index from that noted in paired untreated samples, significant decreases of 20% and 10% were observed upon treatment with 0.1 μg/ml MMC and 0.175 μg/ml ADR, respectively (data not shown).

The values we observed for the mitotic index, with an 18-hr accumulation time in colcemid, suggested that some cells were either not reaching mitosis because of treatment, or were escaping from the mitotic arrest. The extent of escape of CHO cells from the 18-hr colcemid-induced metaphase arrest was therefore measured by using a differential staining technique. In CHO cultures not treated with chemical, a maximum of 20.9±5.4% of the total scorable cells analyzed were determined to be second division cells (Table 27). Cultures exposed to DNA damaging chemicals were found to contain even fewer second division cells; about 5% of the cells analyzed progressed from first to second division in 0.075 μg/ml MMC and 0.175 μg/ml ADR-treated cultures, while treatment with 0.1 μg/ml MMC allowed only about 1% of total cells analyzed to escape (Table 27). Thus, the limited escape and scoring of second division cells is not likely to have affected the results reported herein.

DISCUSSION

Dosimetry Considerations in Far Field Microwave Exposure of Mammalian Cells

The special water bath was designed for far field RFR exposures of in vitro systems. These experiments clearly demonstrate that leaving the circular array of T-25 flasks stationary in the field would be inappropriate, as the cells in different flasks in different positions would be exposed at different SARs. Continuous rotation of the flasks in the RFR field under the rectangular horn is an acceptable way of producing equivalent average exposures in the far field.

In performing in vitro exposures, we did not detect differences in SAR values in medium with cells present, either in suspension or surface attached, as compared with measurements made with no cells present.

In measuring SAR values and subsequently performing experiments, specifying the medium volume is critical since we have clearly observed a difference in measured SAR with changing volume. As the volume increased, the measured SAR decreased under these geometric and RFR field conditions. It is obviously important to perform temperature measurements for SAR calculations at depths in the medium where the cells would be located during exposure.

Dosimetry in RFR studies is very important, and even more complex than that in ionizing radiation or ultraviolet light exposures. The results of this investigation indicate the extra care which must be taken when performing far field in vitro RFR exposures, especially if a given study is ever to be reproduced in another laboratory.

Absence of Mutagenic Interaction Between Microwaves and Mitomycin C or Proflavin in Mammalian Cells

Literature Review

The information available on genetic changes resulting from RFR (microwave) exposure of cells is extremely limited. The focus in this discussion will be on mutation and/or genetic effects, since chromosomal aberration induction studies after RFR (and chemical) exposure are dealt with in another report from our laboratory (Kerbacher et al., 1987), as are sister chromatid exchanges after RFR (and mitomycin C) (Ciaravino et al., 1987), and DNA repair (after UV damage) (Meltz et al., 1987).
In the available literature, the frequencies of exposure vary, as do the biological systems assayed. Technical details provided have been inconsistent; dosimetry is often not presented (or was not performed); temperature data and experimental procedures are inadequate (or were inadequately described); and statistical treatments can be lacking. These qualifications being accepted,however, microwave effects have been investigated in bacteria, yeast, fungus, tradescantia, and mice.

In the bacterial studies, Blackman et al. (1976) irradiated E. coli strain WWU with continuous wave RFR at 1.7 and 2.45 GHz. At power densities of 10 and 50 mW/cm^2 (corresponding to SARs of 15 and 70 mW/cm^2 for the 2.45 GHz exposure [far field]), and at an intensity of 88 V/m for near field exposure at 1.7 GHz (corresponding to an SAR of 3 W/kg),Blackman et al. (1976) reported no increase in mutation for a 3-to 4-hr exposure at a temperature of 35°C. Dutta et al. (1980) exposed a panel of Ames Salmonella tester strains to different temperatures (by convection heating) for induction of mutation, but only studied RFR effects on wild type (pol A^+) and repair deficient (pol A^-) E. coli. The exposures were pulsed wave (1000 Hz) at the frequencies of 8.6, 8.8, and 9.0 GHz. The duty cycle was 0.001; and the power densities reported were 1, 10, or 20 mW/cm^2. The data indicate that the repair deficient strain is more sensitive than the wild type to both elevated temperature and microwave radiation (with an associated elevated temperature). This evidence is only circumstantial as to a mutation effect; the temperature dependence of the relative survivals may, as discussed by the authors, depend on some other mechanism.

Blevins et al. (1980) did expose the Salmonella typhimurium tester strains to 2.45 GHz RFR, as well as to increased temperature. They used a conventional microwave oven, with a stated power density of 5100 mW/cm^2; tubes were said to be located at five positions which gave similar radiation exposures when simultaneously exposed (but it was not stated how this similarity was determined). Exposure times varied from 2 to 23 sec. Temperature controls were performed at the maximum temperature achieved by the RFR heating. For exposures up to 6 sec, where the maximum temperature achieved was 58°C, no mutations were observed. An increased number of mutants were observed at longer exposure times, with increasingly high temperatures. No SAR was reported, but the power density (and heating rate) was extremely high. The authors' data indicate that, for comparable levels of survival (greater than 75%) at equivalent temperatures (greater than 65°C) and exposure times, the number of RFR-induced mutant colonies exceeds the number of convection-heating-induced mutant colonies. Dardalhon et al. (1981) exposed Rec (+/-) repair-deficient strains of E. coli K-12 bacteria,

as well as the WP2 uvrA trp⁻ strain of E. coli B/r, at distances from 2 to 30 mm away from the outlet of a waveguide. The frequencies studied included 9.4, 17, or 70-75 GHz. The power at the outlet was 50 (17 GHz) or 60 mW/cm^2; when a biological indicator was used, the temperature was said to increase by no more than 5°C at any frequency. The SARs were measured to be 9 (70-75 GHz), 23 (9.4 GHz), or 28 (17 GHz) W/kg. After an exposure of 30 min, no difference in survival was found between the repair-deficient and wild-type bacterial strains. Above temperatures of 50°C, using convection heating, a differential response could be demonstrated. When similar exposure experiments were performed at 70-75 GHz or 17 GHz for 30 min with the tryptophan dependent E. coli B/r strain, no mutation to tryptophan independence was observed.

Investigations in yeast were performed by Dutta et al. (1979) and Dardalhon et al. (1985). The former exposed the diploid D4 strain of Saccharomyces cerevisiae, and looked for mitotic recombination and mitotic reversion after exposure to 2.45 GHz continuous wave RFR and 9.0 GHz pulsed wave RFR (1000 Hz; 0.001 duty factor). The exposure time was 2 hr at 29°C or 30°C. When the cells were exposed at power densities ranging from 1.0 to 45 mW/cm at 9.0 MHz, and also at power densities of 1, 5, and 45 mW/cm^2 at seven frequencies ranging from 8.5 to 9.6 GHz, no mutagenic activity was detected. At the highest power level, a 12°C increase in temperature was reported. Daldalhon et al. (1985) exposed haploid Saccharomyces cerevisiae to 9.4 GHz (50 mW/cm^2, 21 W/kg at 5 mm) and 17 GHz (60 mW/cm^2, 24 W/kg at 5 mm) RFR. After one 330-min exposure at power densities up to 60 mW/cm^2, no evidence was found of the induction of cytoplasmic 'petite' mutants.

The effects of microwaves on different strains of the fungus Aspergillus have been reported by Baranski et al. (1976), Mezykowski et al. (1980), and Dhahi et al. (1982). When Baranski et al. (1976) irradiated A. Nidulans with 2.45 GHz RFR (no exposure details available) at power densities in the microwatt range (no data) for 10-240 min, no mutagenic effects were observed. Mezykowski et al. (1980) also exposed A. Nidulans at 2.45 GHz, for 1 hr at a power density of 10 mW/cm^2. The exposures were both continuous wave and pulsed wave (600 pps, 1 μs), at room temperature. The temperature achieved upon RFR exposure, and that of their temperature controls, was said to be 28°C. The cells were exposed both as conidia, which are "rather inactive metabolically," and also after DNA replication had been initiated by a preincubation. The authors reported no change in the number of morphological mutations in either case due to the RFR exposure. These authors also went to the extent of studying a temperature sensitive mutant of the A. Nidulans. When exposing this mutant under the same conditions, with the medium temperature increasing from 40° to 44°C, they also saw no mutations due to the RFR. Dhahi et al. (1982) examined the effect of 8.7175-GHz continuous wave radiation in the free field of the near zone on A. Amstelodami. Conidia were exposed for 3 or 6 hr at 2.09 mW/cm^2. The temperature at onset of exposure ranged between 28 and 30°C. After 3 hr of exposure, the temperature had increased by 2.2°C; after 6 hr, by 2.4°C (relative to the control sample). The endpoints studied included both morphological mutation and a biochemical marker, 8-azaguanine resistance. The authors did not see a statistically significant induction of mutation in any of their experiments.

Genetic effects of RFR on Drosophila melanogaster have been examined by Hamnerius et al. (1979) and Marec et al. (1985). The former exposed

Drosophila melanogaster embryos in water to continuous wave 2.45-GHz radiation; the endpoint studied was a mutation that caused a shift in eye pigmentation. The exposure was at a distance of 0.4 m from an antenna aperture at the end of a waveguide. The temperature was said to be "kept at 24.5°C during the whole irradiation period"; but where the temperature had been measured was not stated. Because of a later statement by the authors that "their thermal effects can be neglected," we must presume that the constant 24.5°C temperature was in the liquid in which the embryos were exposed. The average SAR of the sample was reported as 100 W/kg; the sample container (Teflon) was inside a larger Plexiglas container with flowing thermostatically controlled water. The exposure time was 6 hr. The authors report that they did not see a mutagenic effect under these conditions. Marec et al. (1985) exposed males of a strain of Drosophila melanogaster to continuous wave 2.375 microwave radiation in a sample holder within a waveguide. The power densities and times of repeated exposures on 5 days were: 15 W/cm^2 for 60 min; 20 W/cm^2 for 10 min; or 25 W/cm^2 for 5 min. The endpoint studies were a sex-linked recessive mutation. The flies were non-anesthetized when exposed in a perforated polyethylene tube. The authors reported a high initial mortality for the males in each exposure group. They did not observe a statistically significant increase in the number of sex-linked recessive lethals.

The genetic effects of microwave radiation in mice have been studied by Varma et al. (1975), Goud et al. (1982), and Saunders et al. (1983). In each of these studies, the endpoint for mutagenicity was dominant lethal mutation. This standard assay for mutagenicity in chemical studies suffers, in the sense of the mutagenicity discussion herein, from having chromosome aberrations as one of its known causes. Varma et al. (1975) performed a partial-body exposure, in that only the testes of anesthetized Swiss male mice were irradiated with 2.45 GHz continuous wave irradiation. Varma et al. (1975) reported a statistically significant increase in a "mutagenicity index," but only for mice exposed to a single exposure at 100 mW/cm^2 for 10 min. No SAR was reported, and the temperature reached in the testes was not reported. Interestingly, of 15 mice exposed, 5 died. Goud et al. (1982) exposed Swiss male mice whole body to continuous wave 2.45-GHz radiation from a diathermy unit. The exposure was at a power density of 170 mW/cm^2 for 70 sec. An increase of dominant lethal mutations was reported, as was an increase in another index of mutagenicity, sperm-shape abnormality. No SAR was reported, and no temperature measurements were mentioned. In contrast to these studies, Saunders et al. (1983), who exposed only the lower halves of adult male C3H mice, did not see a mutagenic effect on the germ cells using the dominant lethal assay. These authors exposed the mice in a waveguide system to 2.45-GHz radiation for 30 min (the mode is presumably CW, although not so stated). Saunders et al. (1983) reported a mean half-body SAR of 43.4±1.1 W/kg; and a rectal temperature, at the end of exposure, of 41.5°C (as compared to 34.1°C for sham-exposed animals). This information would lead one to expect that the positive results reported by Varma et al. (1976) and Goud et al. (1982) might be related to temperature increases in the testes (unmeasured), due to the high power densities employed.

Experimental RFR and MMC or Proflavin Studies

The experiments described in this work were designed to examine two hypotheses: First, RFR might act directly on mammalian cells as a genotoxin.

Second, RFR radiation might impact on the action of a known chemical genotoxin in either an additive or antagonistic fashion, because of the multiple steps by which a chemical must enter the cell, pass through the cytoplasm and the nucleoplasm to reach the DNA, interact with the DNA, and have its damage be (or not be) repaired in such a way as to be toxic and mutagenic.

The results (summarized in Tables 9 and 11) clearly indicate that the RFR exposure by itself, under these moderate exposure conditions, was not mutagenic.* Further (as indicated by the data summarized in Tables 12 and 16), no synergistic or antagonistic mutagenic action is present as a result of simultaneous pulsed wave 2.45-GHz RFR and MMC or RFR and proflavin treatment, even though sufficient power density and SAR values were achieved to cause an increase by several degrees in medium temperature. The power density and SAR values were higher than recommended in guidelines for maximum exposure previously existing for this frequency (10 mW/cm^2 power density and 4 W/kg SAR), and are higher still than recently adopted guidelines (American National Standards Institute, 1982). In terms of biohazard concerns, these observations can be comforting. The results also appear to be in accord with the observations already described in other systems, where the mutagenicity of microwave radiation appears to occur only in association with a measured (or likely) temperature increase.

In performing these experiments, it became clear to us that the water-bath exposure system designed for these studies was retarding the temperature increase in the medium in the exposure flasks; the system was acting as a heat sink. The resulting temperature increase, to a maximum of 40°C, is probably not the highest temperatures which could be achieved for the power density and SAR applied. RFR bioeffects could therefore still be possible at these exposure values, but might require a higher temperature. This hypothesis will be explored with a new exposure system allowing for higher temperatures at these and other power density and SAR exposure values.

Effects of RFR and Simultaneous Exposure with Mitomycin C and Adriamycin on the Frequency of Sister Chromatid Exchanges in CHO Cells

The first hypothesis of this study, the possible genotoxic action of 2.45 GHz RFR on CHO cells, was approached by examining the effect of RFR exposure on the frequency of SCEs in an in vitro mammalian cell system. When the effect of RFR exposure was compared with the SCE frequency of cells incubated at 37°C (Table 21: Row E vs. A), no significant difference between

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* In this assay, a statement of even weak mutagenicity would require the induced mutation frequency after RFR exposure be twice the spontaneous (37°C control) mutation frequency.

groups in an ANOVA was observed. This comparison indicated that, at the specified field parameters (2.45 GHz, pulsed wave, power density of 49 mW/cm^2, and an SAR of 34 W/kg) -- resulting in a 2.2°C temperature increase in medium temperature to a maximum temperature of 39.2°C -- there was no induction of SCEs by the RFR. Other comparisons, made between TC and 37°C (Table 21: Row C vs. A) as well as between RFR-exposed and TC (Table 21: Row E vs. C), also showed no statistically significant differences ($p>0.05$).

The second hypothesis of this study was to determine whether RFR exposure of cells during a simultaneous treatment with MMC or Adriamycin could cause a change in the frequency in SCEs from that produced by MMC alone. MMC is an anti-neoplastic antibiotic, and interacts with the deoxyribonucleic acid (DNA) of cells by cross-linking DNA strands. MMC has been demonstrated to act as both a mono- and bifunctional-alkylating agent (Vig, 1977). Latt (1974) demonstrated that MMC was capable of increasing the frequency of SCEs multifold at doses which barely increased the frequency of traditional chromosome aberrations. This theory has been confirmed by using doses as low as 3×10^{-8} M (Perry and Evans, 1975; Kato, 1975; Kato and Shimeda, 1975). For this study, the concentration of MMC was experimentally determined to give an approximate doubling of the baseline SCE frequency observed after a 2-hr exposure. Note, however, that the exposure times to MMC differed slightly between experiments, thus inducing variability in SCE induction by MMC. Two concentrations of Adriamycin, 0.45 and 0.58 $\mu g/ml$, were also studied (Table 22).

Statistical analysis of the data for the simultaneous RFR and MMC and RFR and Adriamycin exposures performed in these experiments showed no significant differences between the response of cells to MMC or Adriamycin exposures at 37°C and to the temperature control and RFR conditions. Further statistical analysis demonstrated the SCE frequencies for 37°C, TC, and RFR (in Table 21, column 4) were significantly different from each of the three previous experiments. ANOVA tests were therefore conducted using the data from the first three experiments with no difference in the overall results. Additionally, an ANOVA of Experiment 4 alone resulted in a conclusion similar to that obtained for the other experiments. The conclusion reached was that, although the non-MMC SCE levels for Experiment 4 were elevated, their inclusion in the overall statistical evaluation of the data did not change the conclusions, but merely introduced variability in the data.

Influence of RFR on Chromosome Aberrations in CHO Cells and Its Interaction with DNA Damaging Agents

One objective of this investigation was to determine whether RFR exposure by itself at these levels could induce the formation of chromosome aberrations in CHO cells by mechanisms not attributable to temperature increases. Environmental exposures of humans to RFR are limited to 0.4 W/kg (5-10 mW/cm^2 at this frequency) by current safety guidelines (ANSI guidelines for both environmental and occupational exposures). An extensive analysis comprising over 2,000 mitotic spreads failed to result in any evidence to suggest that RFR exposure resulted in increased frequency of any aberration type scored (Table 23). These results are in contrast with those of Chen et al. (1974), which suggest a nonthermal increase in the frequency of chromosome aberrations resulting from microwave exposure; they state, however, that their data are not

statistically significant. In agreement with Lloyd et al. (1986), one should note, in this investigation, exposure of cells in tissue culture medium at temperatures as high as 40.2°C for 2 hr failed to produce a significant change in the frequency of chromosome aberrations in CHO cells, regardless of whether the increased temperature resulted from microwave irradiation or water bath convective heating (Table 23). These observations are in a more biologically appropriate temperature range than that reached by Alam et al. (1978), who reported thermal induction of chromosome aberrations in CHO-K1 cells at 49°C.

An important feature of this investigation was the simultaneous exposure of CHO cells to both RFR and chemicals known to cause measurable DNA damage. The rationale for this line of investigation was that, even if RFR does not by itself at these exposure conditions directly damage DNA, RFR might still affect the DNA, so that the ability of chemical genotoxic agents to induce aberrations on a chromosome level would be modulated. Two compounds were chosen on the basis of having different mechanisms of interaction with the DNA molecule in producing damage, MMC and ADR. While both compounds were effective in increasing the frequencies of chromosome aberrations relative to untreated CHO cells, there is no evidence to suggest from our study that a nonthermal interaction of RFR occurs as these chemicals damage DNA.

The experimental conditions of this present investigation incorporated an 18-hr colcemid-induced metaphase arrest. This length of arrest period was chosen to ensure that the CHO cells analyzed would comprise a mixed population, representing cells which were in all portions of the cell cycle at the time of exposure. One disadvantage of this protocol was the possibility of escape from metaphase arrest, allowing a cell to traverse the cell cycle a second time, during which chromosome damage could undergo repair prior to second metaphase arrest and subsequent analysis. The extent of metaphase escape was measured for all of the various conditions of this study. Since, in cultures not treated with chemicals as well as in cultures treated with MMC, there was no indication of an alteration in the frequency of chromosome aberrations due to microwave exposure or increased medium temperatures, the outcome of these studies was probably not affected by the inclusion of the second division chromosome spreads. For the ADR and RFR studies, the less than 5% second-division cells cannot account for the temperature-induced increase in total chromosome aberration events.

In summary, 2.45 GHz RFR irradiation alone, for 2 hr under these exposure conditions, was incapable of inducing chromosome aberrations in CHO cells. During simultaneous chemical and RFR exposures, in which two different chemicals were used and in which 37°C and temperature controls were simultaneously performed by use of convection heating, only two statistically significant differences were observed. (From these were derived the two other significant differences indicated in Table 23.) The differences were observed for only one of the chemicals. Although statistically significant, the observed differences are less than 10%, and may not be biologically significant. Additional experiments, at both higher exposure values (SARs) and temperatures, will be performed with new instrumentation to validate these observations. These results do not support the concept of nonthermal genetic effects of microwaves on mammalian cells, since the only statistically significant difference appears at an elevated temperature.

CONCLUSIONS

The conclusions from this series of investigations are twofold: First, pulsed wave RFR at the specified field parameters and moderate power density and SAR, resulting in a temperature increase in the tissue culture medium to no greater than 40°C, does not cause mutation, sister chromatid exchange, or chromosome aberrations in mammalian cell systems. The RFR is not genotoxic under these conditions. Second, when mammalian cells are treated with genotoxic chemicals and simultaneously exposed to RFR under these same conditions, no effect on the level of mutation or SCE induction is caused by the chemicals alone. For chromosome aberration induction, a very small increase was noted for one specific chemical upon RFR exposure; but this change was associated with the increased temperature. These conditions provide an outside safety factor, against genetic hazard, when one considers the possible exposures to simultaneous insults, both in occupational situations and in the everyday environment of the general population.

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FIGURES 1 - 12

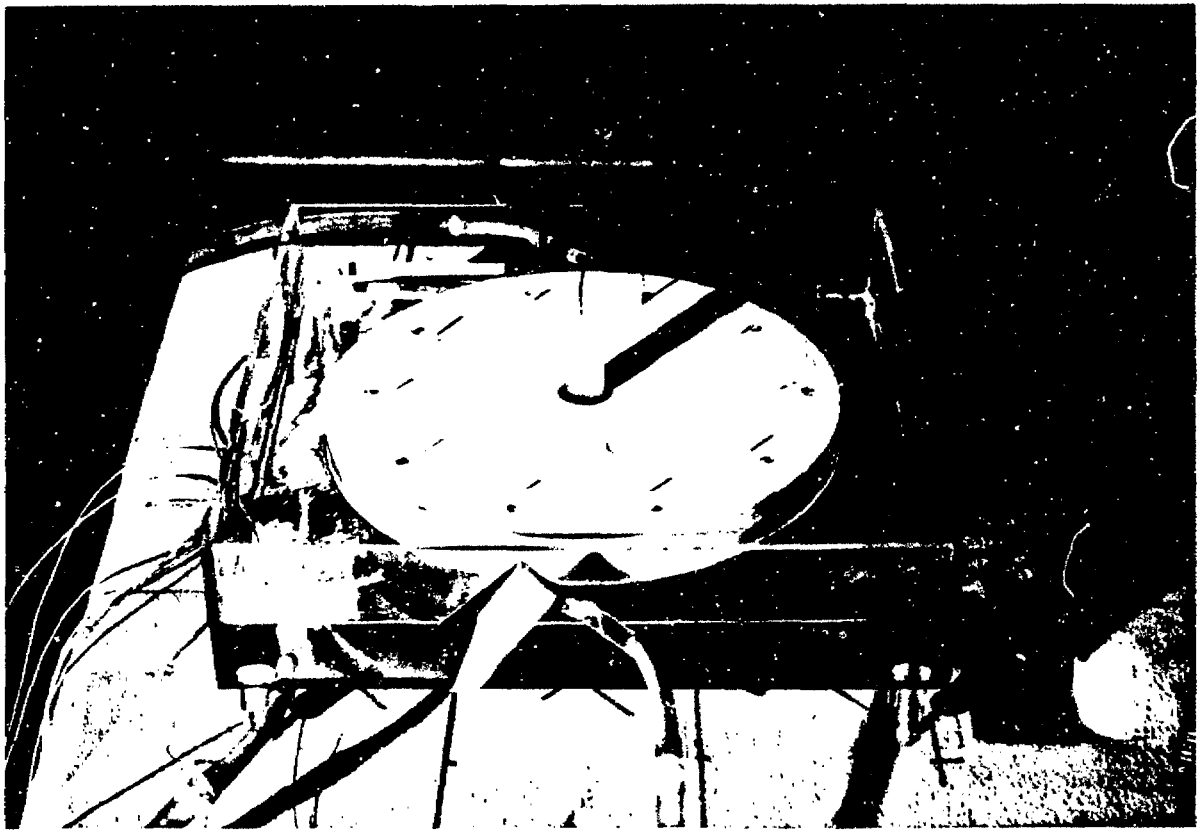

Figure 1. Plexiglas water bath designed to allow continuous rotation of the exposure flasks beneath the antenna horn.

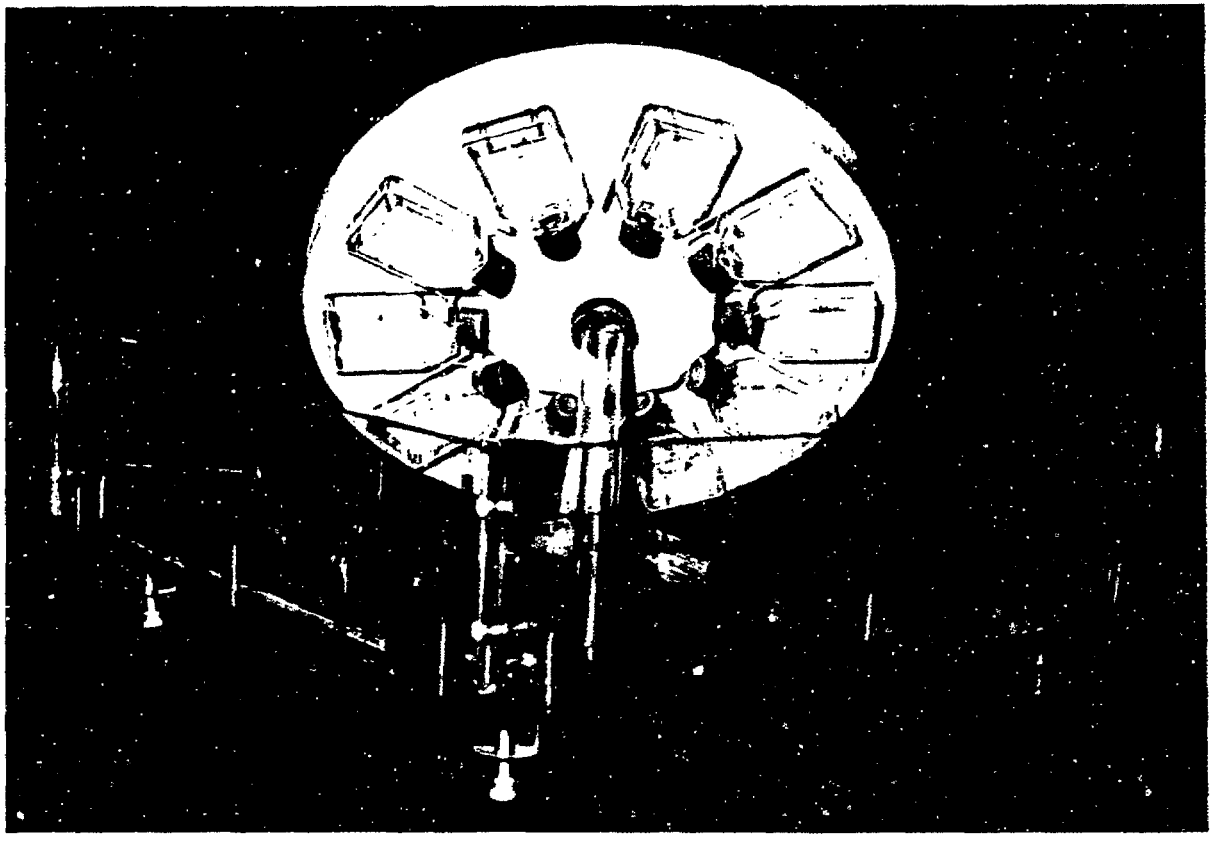

Figure 2. The underside of the Styrofoam wheel which rests on the water surface and is rotated by jets of warm water impinging at an angle on its side.

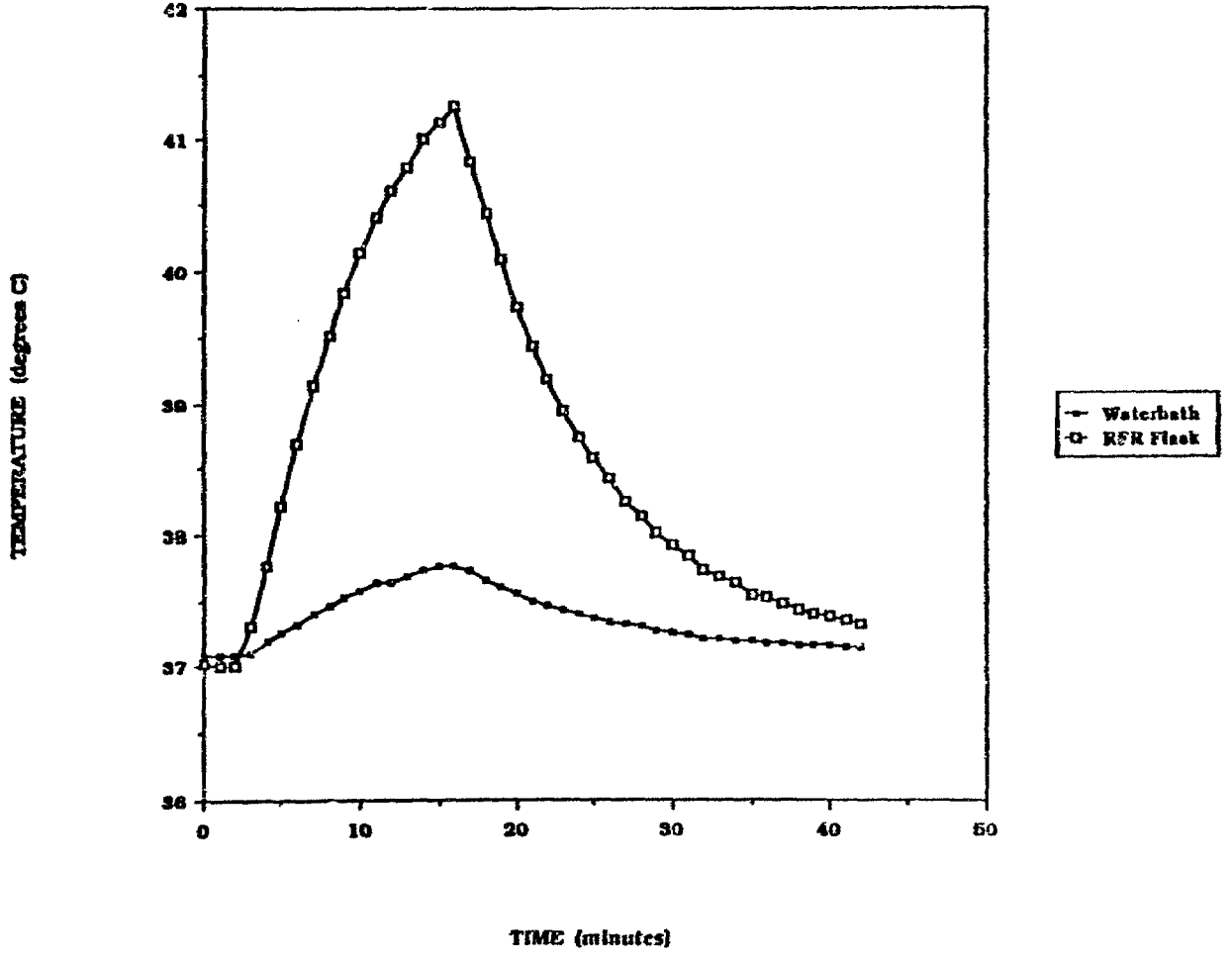

Figure 3. Representative temperatures monitored during heating and cooling periods of SAR determination. Monitoring was performed in medium in exposure flask, and in water in water bath.

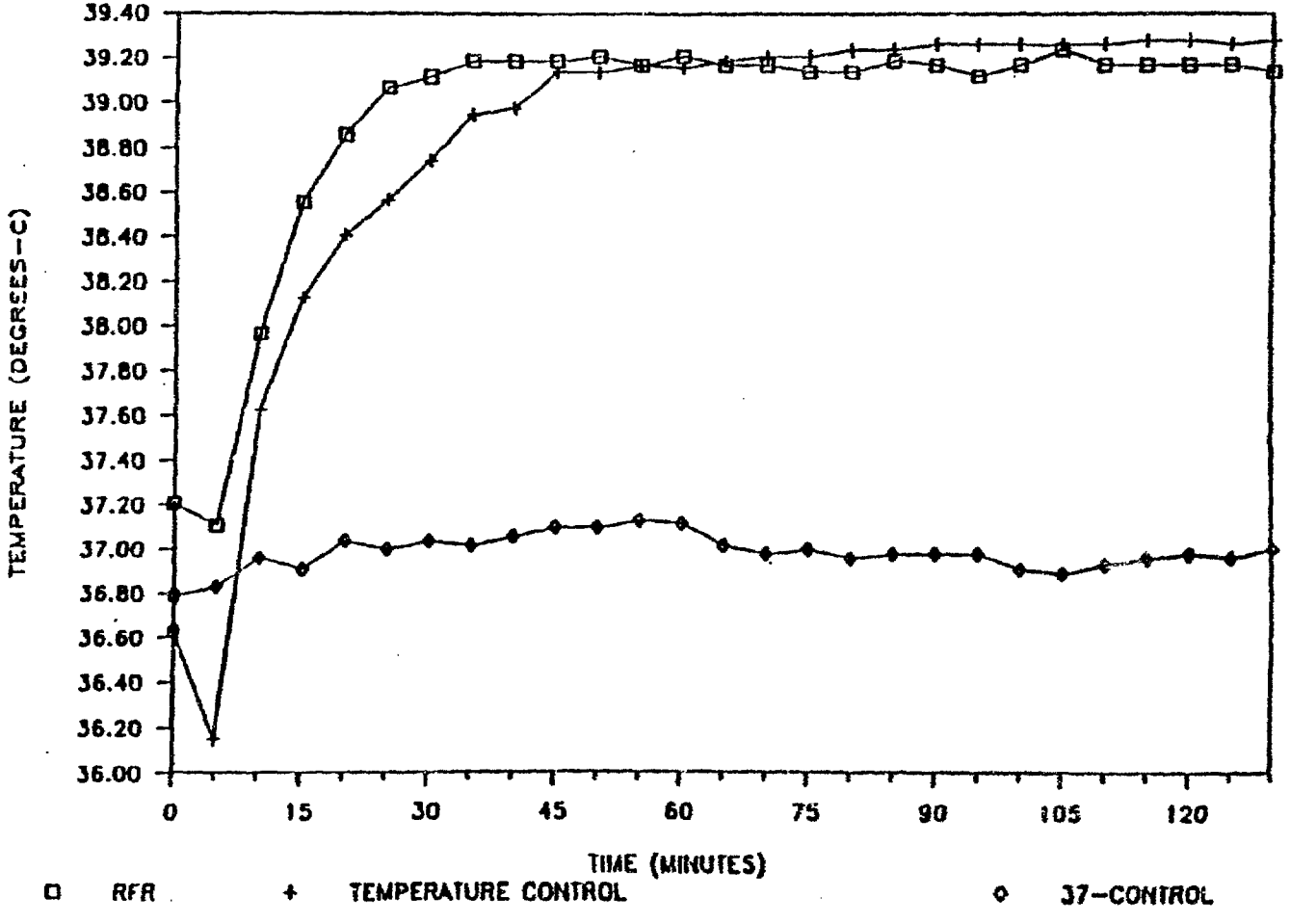

Figure 4. Representative temperatures monitored over a 4-hr exposure period in RFR, TC, and 37°C flasks in mutation experiments.

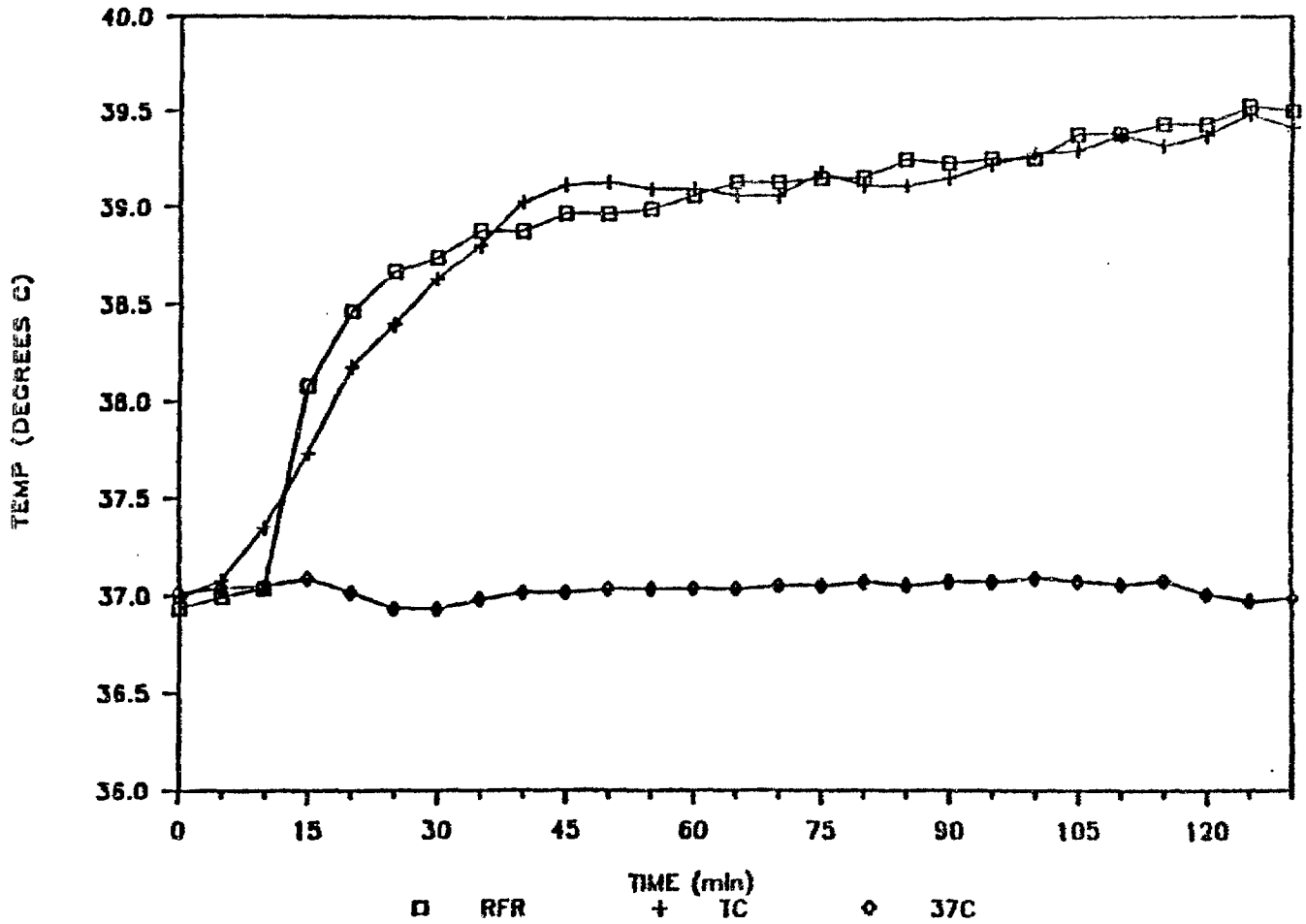

Figure 5. Representative temperatures monitored over a 2-hr exposure period in RFR, TC, and 37°C flasks in SCE and chromosome aberration experiments. The temperatures are values obtained at 5-min intervals; the measurements were actually made every 0.5 min.

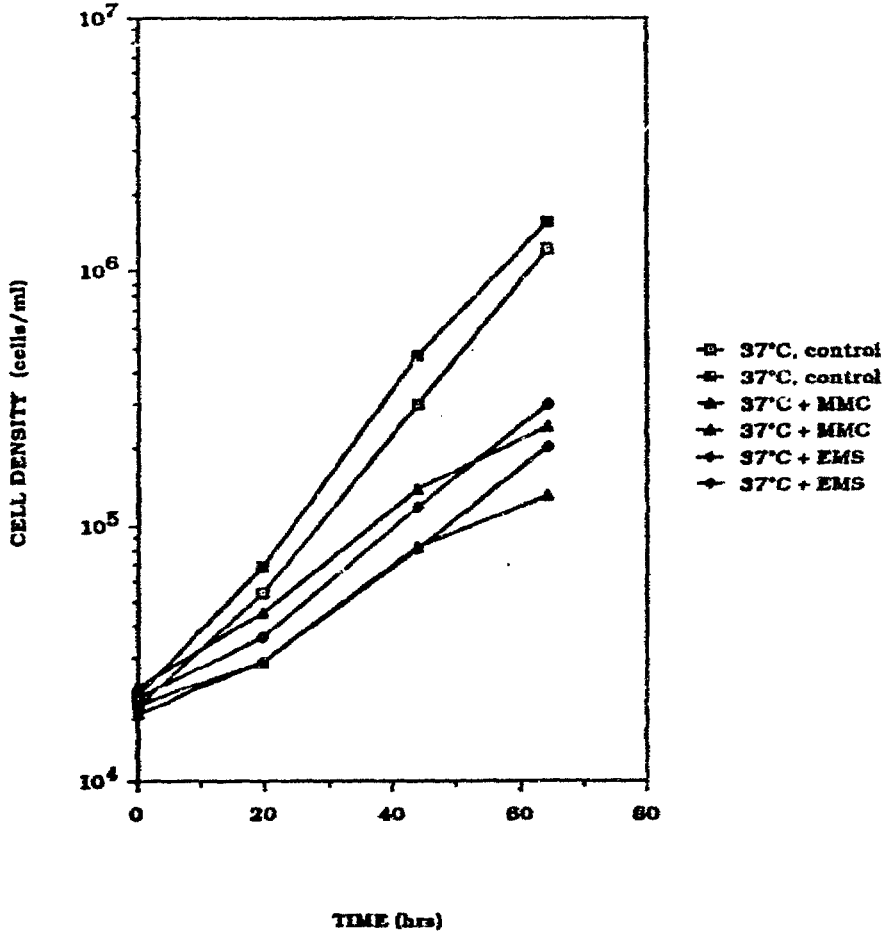

Figure 6. Inhibitory effect on L5178Y cell growth in suspension after treatment with 0.3 µg/ml of MMC or 1 mg/ml EMS at 37°C. Control growth at 37°C is also shown.

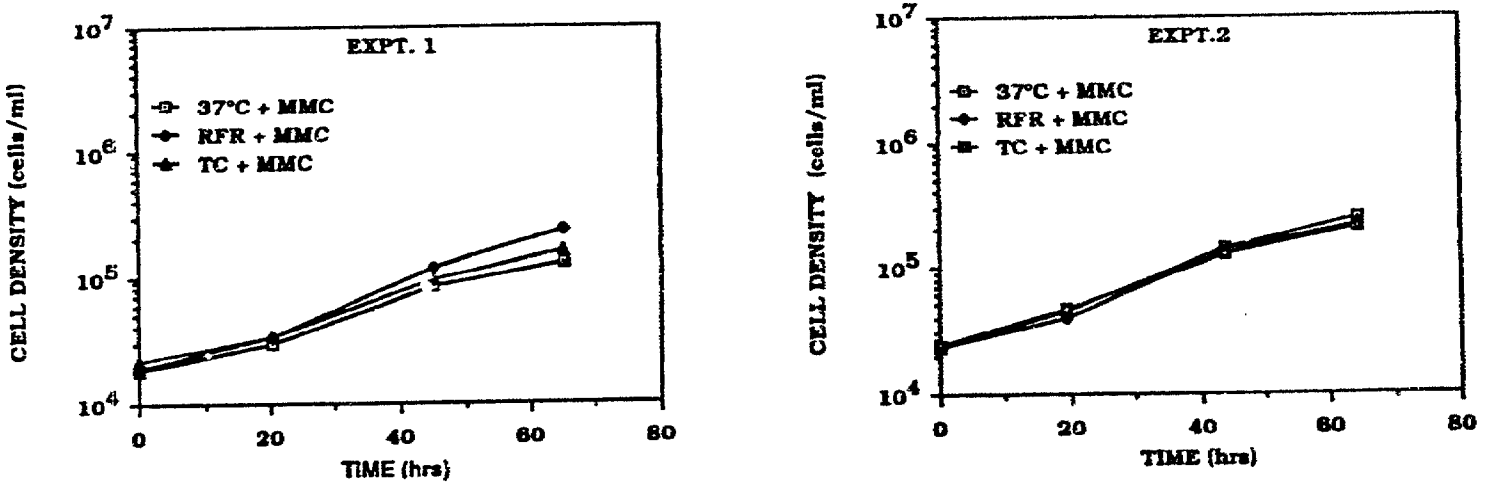

Figure 7. Inhibitory effect on cell growth of MMC at 37°C, or with simultaneous RFR exposure, or under TC conditions. Left chart: Experiment 1; right chart: Experiment 2.

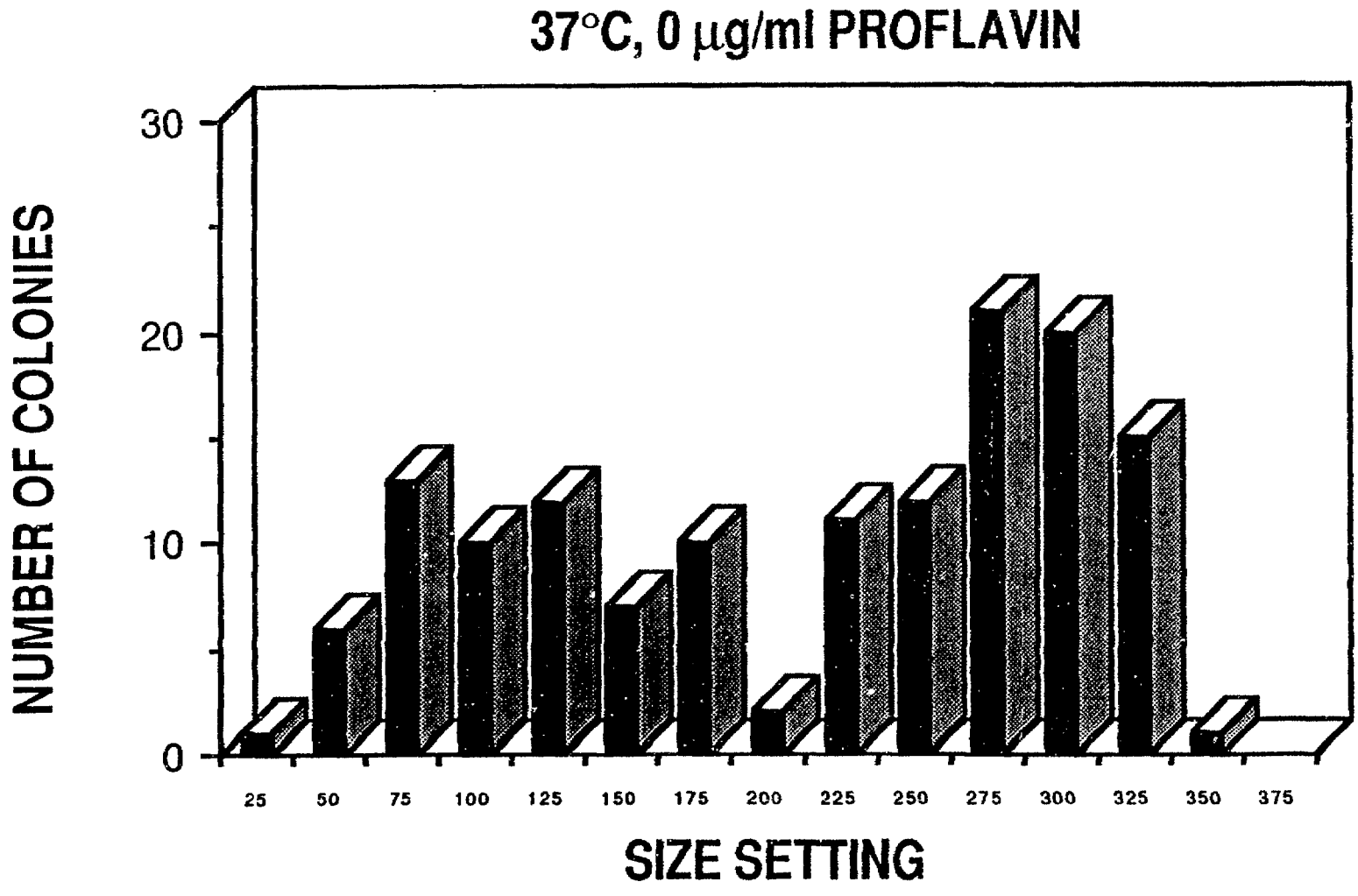

Figure 8. Spontaneous mutant colony size distribution. The number of colonies in a given 25-unit size range setting is presented. The value indicated is the maximum for that size setting range. Note the expansion of the scale of the number of colonies in this chart, as compared to subsequent figures.

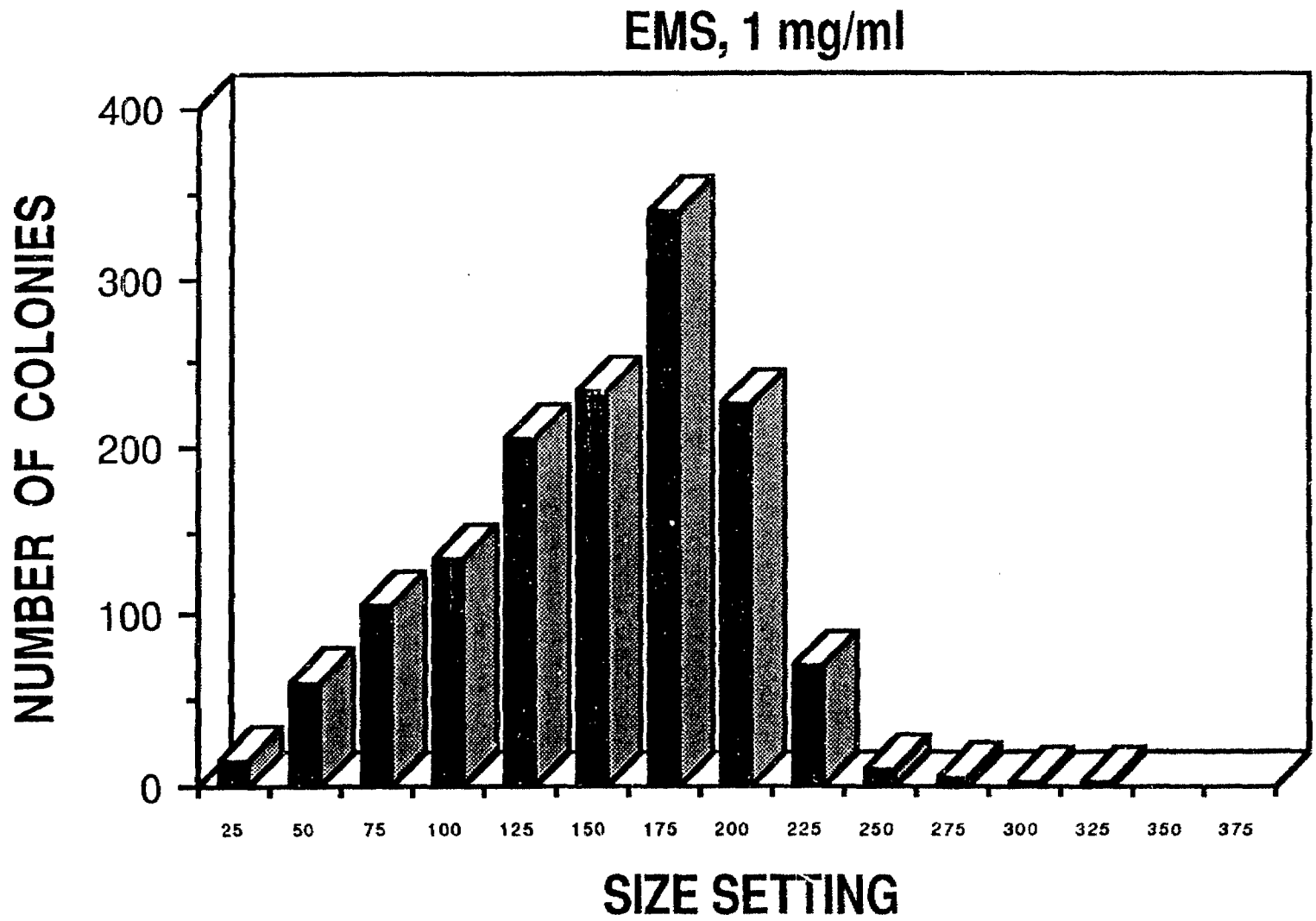

Figure 9. Mutant colony size distribution after a 4-hr treatment with EMS at 1 mg/ml.

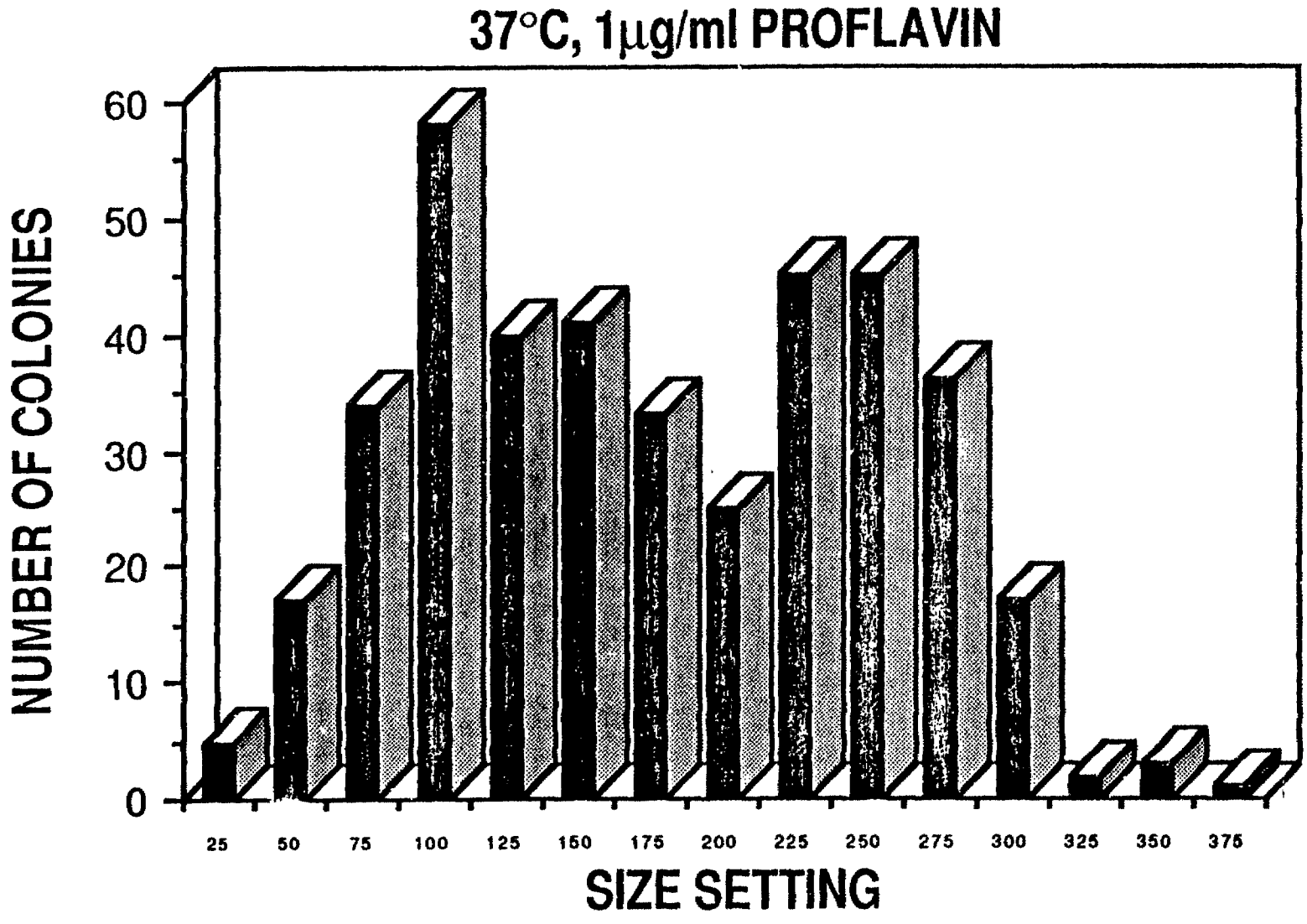

Figure 10. Mutant colony size distribution after a 4-hr treatment with proflavin (1 µg/ml) at 37°C.

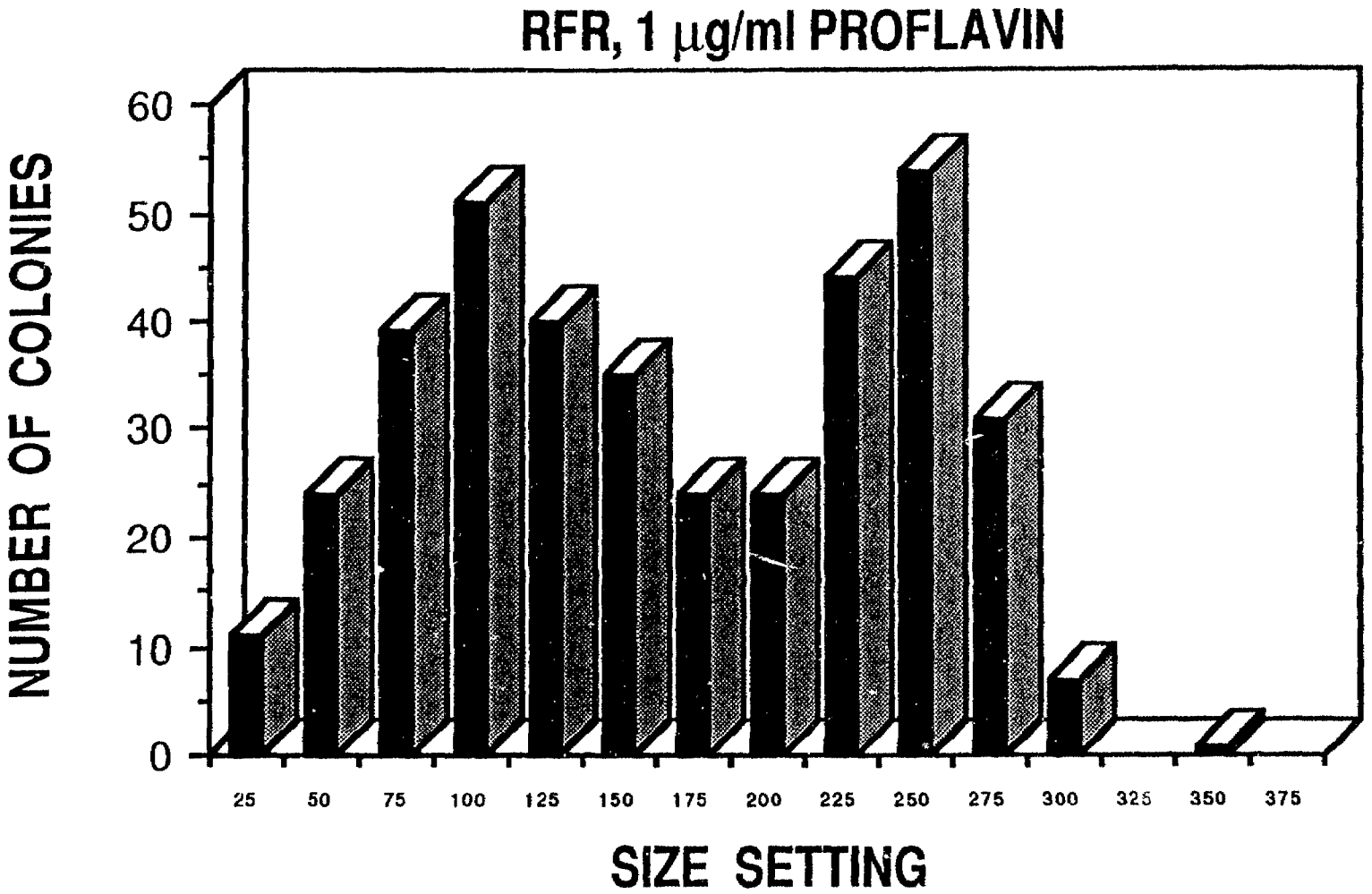

Figure 11. Mutant colony size distribution after a 4-hr treatment with proflavin (1 µg/ml) and simultaneous RFR exposure.

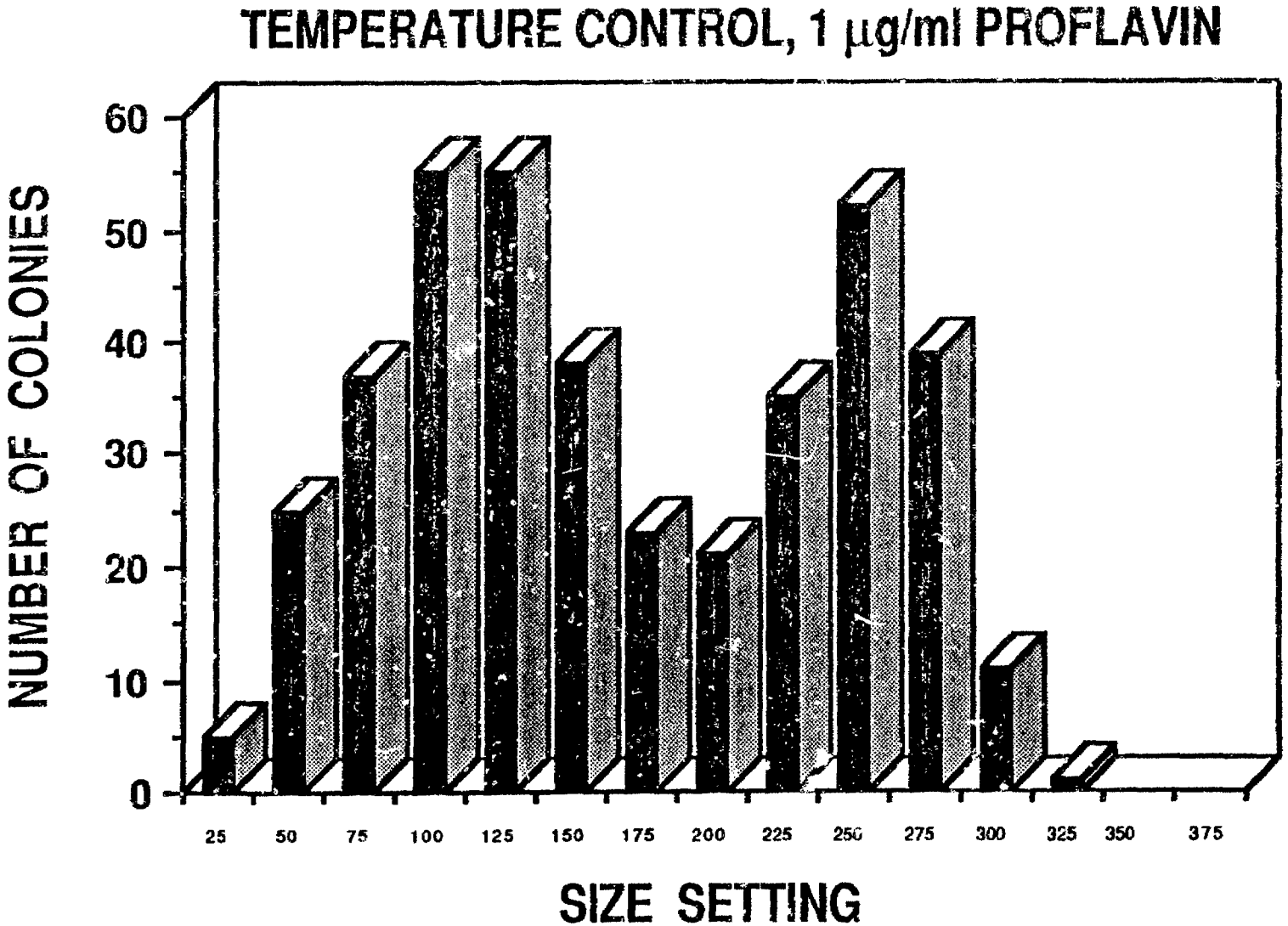

Figure 12. Mutant colony size distribution after a 4-hr treatment with proflavin (1 µg/ml) under TC conditions.

TABLES 1 - 27

TABLE 1. RFR EXPOSURE PARAMETERS

Frequency	2.45 GHz
Mode	pulsed wave
Avg. net forward power	600 W
Power density	49 mW/cm^2
SAR	33.8 W/kg
Repetition rate	25,000 pps
Pulse width	10 μs
Duty factor	0.25
Horn to target distance	1.6 m

TABLE 2. SARs (W/kg) WERE DETERMINED WITH T-25 FLASKS STATIONARY IN THE CIRCULAR STYROFOAM HOLDER IN THE EXPOSURE WATER BATH. FLASKS WERE POSITIONED EITHER PERPENDICULAR OR PARALLEL TO THE HORN LONG AXIS

Flask Long Axis Perpendicular to Horn Long Axis

	Position 2		Position 7		SAR average value
8 ml	18.2 24.0 21.3	21.1 ± 2.9	22.0 25.1 28.2	25.1 ± 3.1	23.2
12 ml	12.4 13.4 9.9	11.9 ± 1.8	9.2 15.4 17.6	14.1 ± 4.4	13.0

Flask Long Axis Parallel to Horn Long Axis

8 ml	50.3 52.7 42.5	48.5 ± 5.3	49.6 63.8 41.9	51.8 ± 11.1	50.2
12 ml	28.2 29.5 29.9	29.2 ± 0.9	30.2 17.3 23.0	23.5 ± 6.4	26.4

Data for two opposing positions obtained simultaneously in three experiments on three different days

TABLE 3. SAR VALUES (W/kg) IN A SINGLE T-25 FLASK IN ROTATION IN THE STYROFOAM HOLDER (POSITION 2) IN THE EXPOSURE WATER BATH*

8 ml		
	22.3[a]	
	29.6	28.2 ± 5.3
	32.6	
12 ml		
	21.1	
	19.1	19.8 ± 1.1
	19.3	

*Individual values listed are for single measurements ([a], or average of 2 measurements) made in different experimental runs on different days.

TABLE 4. SARs (SAR in W/Kg) DETERMINED WITH T-25 FLASKS STATIONARY IN THE CIRCULAR STYROFOAM HOLDER IN THE EXPOSURE WATER BATH: FLASKS POSITIONED BENEATH HORN CORNERS AT AN ANGLE DIRECTED TOWARD THE CENTER

	Position 2		Position 4		Position 7		Position 9	
8 ml								
	35.5		36.3		42.3		42.3	
	32.6	33.3 ± 2.0	42.7	38.1 ± 4.0	44.3	39.3 ± 7.1	38.2	35.4 ± 8.7
	31.8		35.2		31.2		25.8	
	Mean of all positions = 36.5. Greatest difference from mean = 8.8%							
9 ml								
	28.5		37.9		27.6		30.2	
	25.9		29.5		36.5		35.7	
	38.7	31.5 ± 6.1	30.9	34.3 ± 3.9	46.9	36.3 ± 8.7	34.5	33.1 ± 3.7
	37.5		37.9		40.9		36.9	
	Mean of all positions = 33.8. Greatest difference from mean = 7.4%.							
10 ml								
	26.8		26.2		32.6		31.6	
	32.0	29.3 ± 2.6	37.3	31.3 ± 5.6	41.6	35.4 ± 5.4	30.2	29.1 ± 3.1
	29.2		30.3		32.1		25.6	
	Mean of all positions = 31.3. Greatest difference from mean = 13.1%.							

(Cont'd. on facing page)

TABLE 4 (Cont'd.). SARs DETERMINED WITH T-25 FLASKS STATIONARY IN THE CIRCULAR STYROFOAM HOLDER IN THE EXPOSURE FLASKS BENEATH HORN CORNERS AT AN ANGLE DIRECTED TOWARD THE CENTER

	Position 2		Position 4		Position 7		Position 9	
11 ml								
	25.6		27.1		27.3		27.6	
	21.7	23.2 ± 2.1	30.6	28.3 ± 3.0	27.4	26.4 ± 1.6	22.5	26.6 ± 3.7
	22.4		27.2		24.5		29.8	

Mean of all positions = 26.1. Greatest difference from mean = 11.1%

12 ml								
	21.7		26.8		26.7		18.3	
	23.6	22.4 ± 0.9	25.8	24.8 ± 2.3	22.6	25.0 ± 2.1	23.7	23.5 ± 3.6
	21.9		21.6		---		26.1	
	22.4		25.0		25.6		25.8	

Mean of all positions = 23.9. Greatest difference from mean = 6.3%.

TABLE 5. SARs (W/kg) IN 40 ml OF F_{3p} IN A T-25 FLASK AT 2.45 GHz 500-W NET POWER. PULSED MODE AT 25,000 pps, 10-μsec PULSE WIDTH AND 0.25 DUTY FACTOR

	Distance from bottom of Flask			
Experiment No.*	0 mm	4 mm	8 mm	12 mm
1	33.82	45.93	61.59	84.97
2	33.69	45.75	60.50	80.75
3	35.27	43.03	61.64	74.71
4	33.08	42.12	56.13	77.43
Mean	33.97±0.8	44.21±1.67	59.97±2.26	79.47±3.83

* The four values listed at different heights in each experiment (1-4) were obtained from measurements made simultaneously during the experiment.

TABLE 6. COMPARISON OF SAR* (W/kg) WITH AND WITHOUT L5178Y CELLS IN SUSPENSION (WITH ROTATION)

12 ml of F_{3p} - No Cells Present		
	14.81	
	15.33	Mean = 15.4 ± 0.6
	16.07	
12 ml of F_{3p} - Greater than 500,000 cells/ml		
	14.78	
	16.47	Mean = 15.5 ± 0.9
	15.24	

* Each value is the SAR calculated from measurements made in an individual experiment.

TABLE 7. COMPARISON OF SAR* (W/kg) WITH AND WITHOUT CHO CELLS SURFACE ATTACHED (WITH ROTATION)

10 ml of BME - No cells present		
24.46		
27.56	Mean = 26.2 ± 1.6	
26.49		

10 ml of BME - With attached CHO cells		
28.56		
25.18	Mean = 26.7 ± 1.7	
26.46		

* Each value is the SAR calculated from measurements made in an individual experiment.

TABLE 8. MUTATION FREQUENCY/10^6 VIABLE CELLS FOR RFR EXPOSURE AND 37°C, FROM MMC EXPERIMENTS

	Experiment No.				
Replicate No. with RFR	1	2	3	4	5
1	51.7	60.4	109.6	115.5	86.5
2	45.4	108.9	95.6	90.5	108.5
3		32.2	109.0	105.9	119.0
4		22.9	95.1	86.6	88.7
37°C					
1	60.1	41.3	102.4	102.5	74.9
2	58.8	35.0	115.5	99.1	102.4
3		36.8	94.3	99.3	95.9
4		*	81.6	100.1	**
37°C Mean	59.5	37.7	98.4	100.3	91.0

* Designated Outlier excluded from calculation of the mean
** Contaminated sample.

TABLE 9. INDUCED MUTATION FREQUENCY/10^6 VIABLE CELLS FOR RFR EXPOSURE AND TC, FROM MMC EXPERIMENTS

Replicate No. with RFR	Experiment No.				
	1	2	3	4	5
1	-7.8	22.7	11.1	15.3	-4.5
2	-14.1	71.2	-2.9	-9.7	17.5
3		-5.5	10.6	5.6	28.0
4		-14.8	-3.4	-13.6	-2.3
TC					
1	-7.6	11.5	-16.5	---*	---*
2	-2.15	-11.1	-28.7	-10.7	20.7

* Contaminated sample

TABLE 10. MUTATION FREQUENCY/10^6 VIABLE CELLS FOR RFR EXPOSURE AND 37°C, FROM PROFLAVIN EXPERIMENTS

	Experiment No.		
Replicate No. with RFR	1	2	3
1	73.1	145.0	91.8
2	63.3	79.4	---*
3	64.8	83.6	85.3
4	75.4	107.9	74.8
- - - - - - - - - - - - - - -			
37°C			
1	79.9	99.8	83.6
2	81.9	139.8	54.1
3	61.9	108.2	84.8
4	66.7	69 0	89.2
37°C Mean	72.6	104.2	77.9

* Contaminated sample.

TABLE 11. INDUCED MUTATION FREQUENCY/10^6 VIABLE CELLS FOR RFR EXPOSURE AND TC, FROM PROFLAVIN EXPERIMENTS

	Experiment No.		
Replicate No. with RFR	1	2	3
1	0.5	40.8	13.9
2	-9.2	-24.8	---*
3	-7.8	-20.6	7.4
4	2.8	3.7	-3.1
TC			
1	-8.8	12.7	-20.7
2	-7.3	-50.4	- 1.2

* Contaminated sample.

TABLE 12. MEAN INDUCED MUTANT FREQUENCIES PER MILLION VIABLE CELLS FOR FIVE EXPERIMENTS: FOR RFR, 37°C, OR TC WITH MITOMYCIN C (MMC) at 0.3 μg/ml, 0.2 μg/ml or 0.1 μg/ml

	Experiment No. 1	2	3
	(2)0.3 μg/ml	(4)0.3 μg/ml	(4)0.2 μg/ml
37°C + MMC	588.4 ± 99.5	628.8 ± 16.5*	1400.2 ± 121.4
RFR + MMC	762.8 ± 20.5	565.0 ± 53.8*	1388.1 ± 144.7
TC + MMC	823.8 ± 20.5	575.5 ± 31.3	1404.5 ± 195.4

	4	5
	(4)0.2 μg/ml	(4)0.1 μg/ml
37°C + MMC	943.3 ± 79.2	606.9 ± 62.2
RFR + MMC	1464.7 ± 248.4	491.0 ± 59.6*
TC + MMC	818.6 ± 88.1*	616.5 ± 157.4*

* Designated Outlier excluded from calculation of the mean.

( ) = number of replicate independent treatment flasks averaged in each experiment to determine the mean and S.E.

TABLE 13. MITOMYCIN C TREATMENT INDUCED MUTATION FREQUENCY OF L5178Y CELLS EXPOSED AT 37°C

Replicate No.	Experiment No. 1	2	3	4	5
	0.3 μg/ml	0.3 μg/ml	0.2 μg/ml	(0.2 μg/ml)	(0.1 μg/ml)
1	687.6	564.3	1275.2	1049.7	704.9
2	489.2	353.3	1266.5	953.9	429.0
3		588.1	1295.0	715.9	615.6
4		621.0	1763.9	1053.6	678.2

TABLE 14. MITOMYCIN C TREATMENT INDUCED MUTATION FREQUENCY OF L5178Y CELLS EXPOSED TO RFR

Replicate No.	Experiment No. 1 (0.3 μg/ml)	2 (0.3 μg/ml)	3 (0.2 μg/ml)	4 (0.2 μg/ml)	5 (0.1 μg/ml)
1	783.2	477.3	1264.4	772.6	406.0
2	742.4	*	1802.0	1635.5	---**
3		469.9	1137.2	1505.5	622.3
4		634.7	1348.9	1945.2	444.6

* Designated Outlier
** Contaminated sample.

TABLE 15. MITOMYCIN C TREATMENT INDUCED MUTATION FREQUENCY OF L5178Y CELLS EXPOSED TO INCREASED TEMPERATURE BY WATER CONVECTION

Replicate No.	Experiment No. 1 (0.3 μg/ml)	2 (0.3 μg/ml)	3 (0.2 μg/ml)	4 (0.2 μg/ml)	5 (0.1 μg/ml)
1	803.4	475.9	1440.0	---*	773.4
2	844.2	495.8	879.6	719.0	---*
3		570.1	1473.9	994.1	459.6
4		609.3	1824.6	742.8	---*

* Contaminated sample.

TABLE 16. MEAN INDUCED MUTANT FREQUENCIES PER MILLION VIABLE CELLS FOR THREE EXPERIMENTS: RFR, 37°C, OR TC WITH PROFLAVIN (PF) AT 1 μg/ml

	Experiment No. 1 (4)	2 (4)	3 (4)
37°C + PF	162.1 ± 19.8	231.7 ± 12.5*	404.1 ± 47.4
RFR + PF	175.5 ± 16.1*	229.0 ± 10.4	342.4 ± 28.0
TC + PF	160.2 ± 17.6	275.2 ± 57.0	424.2 ± 48.4

( ) = Number of replicates averaged in each experiment to determine mean.

* Outliers (1 in set of 4) not included.

TABLE 17. PROFLAVIN TREATMENT (1 μg/ml): INDUCED MUTATION FREQUENCY OF L5178Y CELLS EXPOSED AT 37°C

Replicate No.	Experiment No. 1	2	3
1	110.4	-5.8*	301.7
2	206.8	253.9	491.6
3	162.3	236.8	345.7
4	168.8	204.5	477.3

* Designated Outlier.

TABLE 18. PROFLAVIN TREATMENT (1 μg/ml): INDUCED MUTATION FREQUENCY OF L5178Y CELLS EXPOSED TO RFR

Replicate No.	Experiment No.		
	1	2	3
1	149.4	204.3	313.1
2	214.1	260.6	279.8
3	654.7*	232.9	373.8
4	162.9	218.1	402.7

* Designated Outlier.

TABLE 19. PROFLAVIN TREATMENT (1 μg/ml): INDUCED MUTATION FREQUENCY OF L5178Y CELLS EXPOSED TO INCREASED TEMPERATURE BY WATER CONVECTION (TC)

Replicate No.	Experiment No.		
	1	2	3
1	185.3	184.9	393.2
2	108.3	256.5	351.2
3	178.2	218.9	385.4
4	169.0	440.4	566.8

TABLE 20. SCE FREQUENCY WITH RESPECT TO MMC CONCENTRATION AND TIME OF EXPOSURE

Time of exposure (hr)	MMC concentration (M)	SCEs per cell ± S.E.M.
0	-----	9.18 ± 0.59
1	1×10^{-9}	10.68 ± 0.52
1	1×10^{-8}	17.30 ± 0.58
1	1×10^{-7}	>50
0	-----	10.80 ± 0.56
2	5×10^{-9}	17.34 ± 0.61
2	1×10^{-8}	20.64 ± 0.85
0	-----	9.18 ± 0.59
3	3×10^{-10}	10.06 ± 0.42
3	3×10^{-9}	17.72 ± 0.92
3	3×10^{-8}	39.46 ± 1.50

TABLE 21. THE FREQUENCY OF SCEs IN CHO CELLS AFTER EXPOSURE TO 2.45 GHz RFR, WITH AND WITHOUT MMC AT A CONCENTRATION OF 1 X 10^{-8}M

	Experiment No.			
	1	2	3	4
	(Number of SCEs Per Cell ± S.E.M.)			
A. 37°C	10.10 ± 0.48	8.10 ± 0.38	9.94 ± 0.48	17.22 ± 0.72
B. 37°C + MMC	18.94 ± 0.60	21.84 ± 0.85	29.48 ± 1.09	25.42 ± 1.01
C. Temperature Control (TC)	10.54 ± 0.42	8.90 ± 0.45	10.84 ± 0.45	16.94 ± 0.77
D. TC + MMC	25.52 ± 0.84	32.36 ± 1.07	32.10 ± 0.94	24.88 ± 0.79
E. RFR	10.90 ± 0.46	9.88 ± 0.39	9.56 ± 0.39	13.78 ± 0.56
F. RFR + MMC	27.02 ± 0.84	27.10 ± 0.91	31.28 ± 1.10	19.22 ± 0.94

TABLE 22. THE FREQUENCY OF SCEs IN CHO CELLS AFTER EXPOSURE TO 2.45 GHz RFR, WITH AND WITHOUT ADR AT CONCENTRATIONS OF 0.45 AND 0.58 μg/ml

	Adriamycin (0.45 μg/ml) Experiment No.			
	1	2	3	4
	(Number of SCEs Per Cell ± S.E.M.)			
Control	7.92 ± 0.36	10.12 ± 0.47	9.48 ± 0.40	8.22 ± 0.37
C-ADR	16.90 ± 0.74	17.38 ± 0.83	13.42 ± 0.80	13.86 ± 0.54
Temp Control	8.72 ± 0.38	8.10 ± 0.40	8.38 ± 0.45	8.94 ± 0.43
TC-ADR	20.90 ± 0.83	15.76 ± 0.61	15.14 ± 0.81	13.74 ± 0.70
RF	8.70 ± 0.39	8.90 ± 0.48	9.06 ± 0.43	8.10 ± 0.44
RF-ADR	17.66 ± 0.93	15.94 ± 0.75	16.18 ± 0.74	13.60 ± 0.56

	Adriamycin (0.58 μg/ml) Experiment No.		
	1	2	3
	(Number of SCEs Per Cell ± S.E.M.)		
Control	9.48 ± 0.56	8.18 ± 0.45	11.0 ± 0.57
C-ADR	19.68 ± 0.83	19.62 ± 1.04	23.06 ± 0.93
Temp Control	8.72 ± 0.42	8.44 ± 0.45	12.38 ± 0.50
TC-ADR	20.76 ± 1.07	17.04 ± 0.75	28.78 ± 1.15
RF	8.90 ± 0.41	9.14 ± 0.46	11.20 ± 0.52
RF-ADR	22.46 ± 1.09	22.06 ± 0.98	23.06 ± 0.93

TABLE 23. INFLUENCE OF RFR AND WATER-BATH HYPERTHERMIA ON THE FREQUENCY OF CHROMOSOME ABERRATIONS IN UNTREATED CHO CELLS

Condition	Aberration type (per 100 cells)				
	tg	tb	sg	sb	f
37°C	1.5 ± 0.3	0.7 ± 0.2	3.5 ± 0.5	1.1 ± 0.3	1.1 ± 0.2
RFR	1.9 ± 0.2	0.8 ± 0.2	3.1 ± 0.4	1.1 ± 0.3	1.1 ± 0.3
TC	1.9 ± 0.3	1.0 ± 0.2	3.5 ± 0.4	1.5 ± 0.4	1.0 ± 0.2

Condition	Aberration type (per 100 cells)				
	cr	min	td	r	d
37°C	0	1.9 ± 0.4	0	0.1 ± 0.1	0
RFR	0	0.8 ± 0.3	0	0.1 ± 0.1	0
TC	0	1.0 ± 0.2	0.1 ± 0.1	0.1 ± 0.1	0

Condition	Aberration summary				
	% Aberrant cells	Aberration events (per 100 cells)	Aberration types (per 100 cells)	Aberrations per cell	Aberrations per aberrant cell
37°C	9.4 ± 0.5	10.1 ± 0.6	10.0 ± 0.6	0.10 ± 0.01	1.07 ± 0.02
RFR	8.0 ± 0.6	9.0 ± 0.8	8.9 ± 0.8	0.09 ± 0.01	1.10 ± 0.02
TC	9.6 ± 0.6	10.1 ± 0.6	10.0 ± 0.6	0.10 ± 0.01	1.05 ± 0.02

The above data represent pooled results from seven experiments comprising 2100 cells in each condition (100 mitotic figures from each of three replicate slides per experiment), and are expressed as mean ± SEM. Abbreviations are: tg-chromatid gap, tb-chromatid break, sg-chromosome gap, sb-chromosome break, f-fragment, cr-complex rearrangement, min-minute, td-terminal chromatid deletion, r-ring, and d-dicentric. RFR-RFR Exposed; and TC-Temperature Control.

TABLE 24. INFLUENCE OF RFR AND WATER-BATH HYPERTHERMIA ON THE FREQUENCY OF CHROMOSOME ABERRATIONS IN CHO CELLS TREATED WITH 0.075 μg/ml MMC

Condition	Aberration type (per 100 cells)				
	tg	tb	sg	sb	f
37°C	7.8 ± 1.1	17.2 ± 3.3	25.5 ± 3.5	3.7 ± 0.3	9.0 ± 2.6
RFR	7.7 ± 2.0	17.2 ± 2.9	23.7 ± 5.4	4.3 ± 1.3	10.2 ± 0.3
TC	6.3 ± 1.5	11.5 ± 3.4	26.2 ± 6.7	2.5 ± 0.8	10.2 ± 2.6

Condition	Aberration type (per 100 cells)				
	cr	min	td	r	d
37°C	1.3 ± 0.3	3.3 ± 0.9	0.3 ± 0.3	0.3 ± 0.3	0
RFR	1.5 ± 0.6	3.7 ± 1.1	0.5 ± 0.3	0	0
TC	1.4 ± 0.6	2.0 ± 0.5	0.5 ± 0.2	0.4 ± 0.2	0

Condition	Aberration summary				
	% Aberrant cells	Aberration events (per 100 cells)	Aberration types (per 100 cells)	Aberrations per cell	Aberrations per aberrant cell
37°C	50.0 ± 1.7	70.0 ± 3.3	68.3 ± 3.3	0.70 ± 0.05	1.39 ± 0.03
RFR	49.3 ± 3.0	70.2 ± 5.2	68.7 ± 5.5	0.70 ± 0.05	1.42 ± 0.03
TC	42.3 ± 2.6	62.2 ± 4.6	60.7 ± 4.6	0.62 ± 0.05	1.46 ± 0.04

Data for 37°C control and RFR-exposed cells represent pooled results from two separate experiments comprising 600 cells per condition (100 mitotic figures from each of three replicate slides per experiment), while TC data comprise a total of 560 cells. The values are expressed as mean ± SEM. Abbreviations are the same as in Table 23.

TABLE 25. INFLUENCE OF RFR AND WATER BATH HYPERTHERMIA ON THE FREQUENCY OF CHROMOSOME ABERRATIONS IN CHO CELLS TREATED WITH 0.1 μg/ml MMC

Condition	Aberration type (per 100 cells)				
	tg	tb	sg	sb	f
37°C	20.7 ± 1.2	20.1 ± 3.5	34.6 ± 2.2	5.7 ± 1.1	8.8 ± 0.8
RFR	18.9 ± 1.6	25.4 ± 3.4	33.4 ± 3.5	6.1 ± 1.3	10.3 ± 1.4
TC	21.9 ± 2.4	25.3 ± 3.4	35.4 ± 1.9	4.3 ± 0.5	9.3 ± 1.5

Condition	Aberration type (per 100 cells)				
	cr	min	td	r	d
37°C	1.4 ± 0.4	7.6 ± 0.4	0.3 ± 0.2	0.8 ± 0.4	0.2 ± 0.1
RFR	4.1 ± 1.3	7.9 ± 1.4	0.3 ± 0.2	0.6 ± 0.2	0.3 ± 0.2
TC	2.2 ± 0.7	5.6 ± 0.8	0.6 ± 0.3	0.3 ± 0.2	0.1 ± 0.1

Condition	Aberration summary				
	% Aberrant cells	Aberration events (per 100 cells)	Aberration types (per 100 cells)	Aberrations per cell	Aberrations per aberrant cell
37°C	62.8 ± 1.5	102.6 ± 3.0	100.1 ± 3.0	1.03 ± 0.03	1.64 ± 0.06
RFR	65.3 ± 1.6	111.1 ± 5.2	106.2 ± 4.8	1.11 ± 0.05	1.70 ± 0.05
TC	64.1 ± 0.7	108.9 ± 5.3	106.2 ± 4.8	1.09 ± 0.05	1.70 ± 0.08

The above data represent pooled results from three experiments comprising 900 cells in each condition (100 mitotic figures from each of three replicate slides per experiment), and are expressed as mean ± SEM. The abbreviations are the same as in Table 23.

TABLE 26. INFLUENCE OF RFR AND WATER BATH HYPERTHERMIA ON THE FREQUENCY OF CHROMOSOME ABERRATIONS IN CELLS TREATED WITH 0.175 μg/ml ADR

Condition	Aberration type (per 100 cells)				
	tg	tb	sg	sb	f
37°C	2.8 ± 0.4	2.8 ± 0.6	3.8 ± 1.0	18.8 ± 1.3	3.8 ± 0.4
RFR	2.4 ± 0.5	2.5 ± 0.6	4.7 ± 1.2	19.3 ± 1.7	3.8 ± 0.8
TC	2.8 ± 0.6	1.5 ± 0.4	4.6 ± 0.8	22.6 ± 1.6	4.4 ± 0.9

Condition	Aberration type (per 100 cells)				
	cr	min	td	r	d
37°C	0.1 ± 0.1	6.9 ± 1.2	0	1.2 ± 0.4	1.3 ± 0.3
RFR	0.5 ± 0.2	6.4 ± 0.8	0	1.6 ± 0.5	2.1 ± 0.3
TC	0.2 ± 0.1	5.9 ± 0.5	0	1.7 ± 0.3	1.3 ± 0.5

Condition	Aberration summary				
	% Aberrant cells	Aberration events (per 100 cells)	Aberration types (per 100 cells)	Aberrations per cell	Aberrations per aberrant cell
37°C	33.8 ± 1.4	43.9 ± 0.8	41.3 ± 1.0	0.44 ± 0.01	1.31 ± 0.05
RFR	35.3 ± 1.2	47.5 ± 1.3*	43.3 ± 1.3	0.48 ± 0.01*	1.35 ± 0.04
TC	36.2 ± 1.5	48.2 ± 1.7*	45.0 ± 1.7	0.48 ± 0.02*	1.34 ± 0.03

The above data represent pooled results from four experiments comprising 1200 cells in each condition (100 mitotic figures from each of three replicate slides per experiment), and are expressed as mean ± SEM. The abbreviations used are the same as in Table 23. *Indicates data statistically different from 37°C values at greater than 95% confidence level by two-way analysis of variance.

TABLE 27. MITOTIC ESCAPE OF UNTREATED AND MMC- OR ADR-TREATED CHO CELLS DURING A SUBSEQUENT 18-HR COLCEMID EXPOSURE

Condition	Chemical treatment			
	None	MMC[a]	MMC[b]	ADR[c]
37°C	16.9 ± 4.6	3.0 ± 3.0	1.0 ± 1.0	4.3 ± 1.2
RFR	20.9 ± 5.9	0 ± 0	0 ± 0	3.3 ± 0.9
TC	18.9 ± 6.2	3.0 ± 2.1	0 ± 0	4.3 ± 1.3

a) 0.075 μg/ml MMC
b) 0.1 μg/ml MMC
c) 0.175 μg/ml ADR

Cultures were incubated in the presence of colcemid (0.2 μg/ml) and BrdUrd (10 μM) for 18 hr at 37°C, as described in the Methods section. Data for untreated cells represent pooled results from two separate experiments, comprising 700 cells (100 scorable mitotic figures from each of seven replicate slides); data for chemical-treated cells represent results of analysis of 300 cells per condition (100 scorable mitotic figures from each of three replicate slides). Data are presented as mean ± S.E.M. Abbreviations used are the same as in previous tables.