United States
Environmental Protection
Agency

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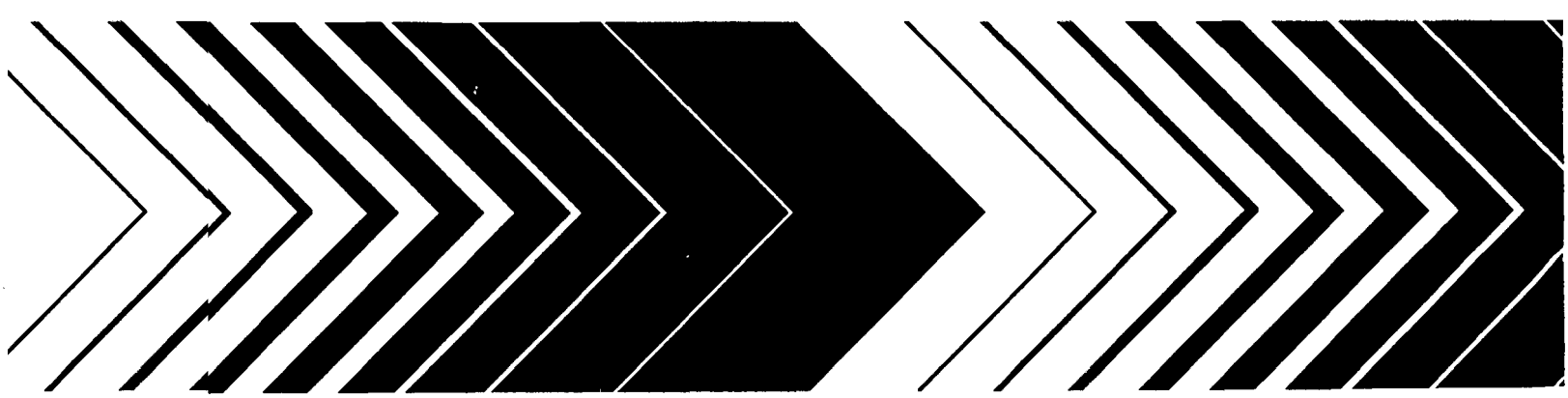
EPA/600/4-90/034
December 1990

Research and Development

EPA

Evaluation of Exposure Markers

Final Report



EVALUATION OF EXPOSURE MARKERS
Final Report For September 21, 1988 To September 20, 1990

by

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EPA Contract Number 68-C8-0069

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ABSTRACT

A novel microgel electrophoresis assay has been developed for directly evaluating, in individual cells, the frequency of single strand DNA breaks and/or alkali-labile sites. This technique, called the single cell gel electrophoresis (SCG) assay, requires the processing of only a few hundred to a few thousand cells. This requirement for an extremely small number of cells makes it possible to evaluate the level and intercellular variability of DNA damage induced by genotoxic agents in virtually any eukaryote cell population.

The primary purpose of this contract has been to determine the suitability of this technique for detecting DNA damage induced by potentially genotoxic pollutants either in cells sampled from various organs of rodents or in cells sampled from humans. In conducting this project, the focus of the research has been on: (i) evaluating the specificity and sensitivity of the technique by determining the magnitude and kinetics of DNA damage induced in cultured mammalian cells (e.g., mouse or human peripheral blood leukocytes, Chinese hamster ovary cells, rodent hepatocytes) by a variety of genotoxic and nongenotoxic chemicals; (ii) developing appropriate methods for isolating individual cells from organs (e.g., blood, brain, liver, spleen, testis, bone marrow, lung) of rodents; (iii) evaluating the kinetics of DNA damage induced in various organs of male mice by a representative environmental genotoxic pollutant; (iv) examining the applicability of the assay to peripheral blood leukocytes obtained from humans exposed to genotoxic agents; and (v) comparing the levels of DNA damage in the organs of mice collected at an EPA superfund site and a concurrent control site.

In many of these studies, the induction of DNA damage was investigated using three representative environmental genotoxic pollutants -acrylamide, trichloroethylene and dimethylbenzanthracene. Based on the results obtained, this technique will provide, with greater sensitivity than any other method currently available, data on the induction and persistence of organ-specific levels of DNA damage resulting from environmental exposure to genotoxic pollutants.

This report was submitted in fulfillment of contract number 68-C8-0069 under the sponsorship of the U.S. Environmental Protection Agency. This report covers a period from September 21, 1988 to September 20, 1990, and work completed as of August 1, 1990.

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The development and application of the SCG technique to a variety of in vitro and in vivo (laboratory animal, free-living animals, human) systems and the wealth of data collected could not have been obtained without the involvement and technical expertise of a number of talented individuals whose participation far exceeded the required scope of the project. These individuals include: P.W. Andrews, D. Croom, B. Nascimbini, and M. Phillips at ILS, and Dr. O. Hirai, a visitor in residence for a year from Fujisawa Pharmaceutical Company, Osaka, Japan. The application of the SCG technique to the in vitro rodent hepatocyte culture system is entirely due to Dr. Hirai's abilities and persistence. The U.S. Army Biomedical Research and Development Laboratory, Ft. Detrick, Frederick, MD provided partial support to this research under Interagency Agreement No. RW21934132-0 (U.S. Army Project Order No. 89PP9951) with the U.S. Environmental Protection Agency.

1.0 INTRODUCTION

The ecological impact and risks to human health (e.g. cancer, heritable mutations) associated with exposure to environmental pollutants are exceedingly difficult to evaluate. Adverse exposure situations resulting from the improper disposal of hazardous wastes are generally identified by epidemiologic investigations or by analytical techniques characterizing the levels of known pollutants. However, because of the generally limited sample sizes intrinsic to studies involving point sources of pollution, the inaccurately defined exposure conditions and, for cancer induction, the long lag time before expression, epidemiologic studies are of limited value in preventing adverse health outcomes resulting from localized pollution. Analytical techniques, while of great value in delineating the kinds of pollution present, do not provide insight into the biological hazards associated with complex mixtures as they interact or are acted upon by various environmental pathways (Vaughan, 1984).

An additional approach for assessing the possible environmental consequences of hazardous waste pollution involves the assessment of genotoxic damage, cytotoxic damage and other ill health effects in sentinel organisms. In marine environments, sea urchins, mussels, benthic worms, and various species of fish (Kligerman and Bloom, 1976; Black, 1984) have been used (or proposed for use) as organisms with which to monitor for adverse effects resulting from toxic pollution. In terrestrial environments, birds (Hill and Hoffman, 1984) and plants, particularly the Tradescantia stamen hair system (Schairer et al., 1978), have long been used to assess toxic levels of environmental pollution. More recently, interest has focused on mammalian species living in close proximity to man. Rowley et al. (1983) published data demonstrating the demographic impact of toxic wastes at Love Canal, New York, on resident meadow vole populations. Nayak and Petras (1985) reported an association between proximity to industrial areas and increased levels of genotoxic damage in feral house mice. More recently, Tice et al. (1988) reported an increased frequency of genotoxic damage among rodents collected at a hazardous waste site in New Jersey.

Techniques which permit the sensitive detection of DNA damage are useful in studies of toxicology and carcinogenesis. Since the effects of toxicants are often tissue and cell-type specific, it is important to develop techniques which can detect DNA damage in a variety of organs or, more importantly, in individual cells obtained from various organs. Currently, the three most commonly used in vivo methods for ascertaining the ability of chemicals to induce DNA damage involves the scoring of chromosomal aberrations, micronuclei and/or sister chromatid exchanges in proliferating cell populations (Tice and Ivett, 1985; Allen, 1988; Tice, 1988), the detection of DNA repair synthesis (so-

called unscheduled DNA synthesis or UDS) in individual cells (Furihata et al., 1984; Mirsalis and Butterworth, 1980; Mirsalis, 1988) and the detection of single-strand DNA breaks and/or alkali labile sites in pooled cell populations (Petzold and Swenberg, 1976; Cavanna et al., 1980; Barbin et al., 1983; Bermudez, 1988). While providing information about damage in individual cells, the cytogenetic techniques are of limited value because of the necessity for proliferating cell populations and because the DNA damage must be processed into microscopically visible lesions. The autoradiographic technique is based on the excision repair of DNA lesions, as demonstrated by the incorporation of tritiated thymidine into DNA repair sites. While providing information at the level of the individual cell, the technique is technically cumbersome and not all DNA lesions are repaired with equal facility. For example, the repair of O^6-methylguanine is largely independent of excision repair processes (Tice and Setlow, 1984). Furthermore, based on statistical grounds, the technique is insensitive to weak mutagens (Margolin and Risko, 1988). Biochemical techniques to evaluate DNA damage directly such as alkaline elution or alkaline gel electrophoresis appear to circumvent some of the problems associated with the other two techniques (Petzold and Swenberg, 1976; Cavanna et al., 1980; Larsen et al., 1982; Sina et al., 1983; Barbin et al., 1983; Bermudez, 1988). However, the use of pooled cells eliminates an evaluation of damage in small target tissues and ignores the importance of intercellular differences in response. Furthermore, possible artifacts associated with the technique (Taningher et al., 1987) and technical limitations associated with experiment to experiment variability (Doerjer et al., 1988) have limited the general use of this approach.

Biochemical approaches for detecting DNA damage directly in single cells have been developed but have not been applied formally to in vivo research. Rydberg and Johanson (1978) were the first to directly quantitate DNA damage in individual cells by lysing cells embedded in agarose on slides under mild alkali conditions to allow the partial unwinding of DNA. After neutralization, the cells are stained with acridine orange and the extent of DNA damage quantitated by measuring the ratio of green (indicating double-stranded DNA) to red (indicating single-stranded DNA) fluorescence using a photometer. To improve the sensitivity for detecting DNA damage in isolated cells, Ostling and Johanson (1984) developed a microgel electrophoresis technique. In this technique, cells are embedded in agarose gel on microscope slides, lysed by detergents and high salt and then electrophoresed under neutral conditions. Cells with increased DNA damage display increased migration of DNA from the nucleus towards the anode. The migrating DNA is quantitated by staining with ethidium bromide and by measuring the intensity of fluorescence at two fixed positions within the migration pattern using a microscope photometer. However, while the neutral conditions for lysis and electrophoresis permit the detection of

double-stranded DNA breaks, they do not allow for the detection of either single-stranded breaks or alkali-labile sites. Since many agents induce from 5 to 2000 fold more single-stranded breaks than double-stranded ones, neutral conditions are clearly not as sensitive as alkaline conditions in detecting DNA damage.

Recently, Singh et al. (1988) introduced a microgel electrophoretic assay capable of detecting DNA single strand breaks and/or alkali labile sites in individual cells. The importance of this assay lies in its ability to detect intercellular differences in DNA damage/repair, and in the requirement for extremely small cell samples. Furthermore, the single cell gel (SCG) technique appears to be quite sensitive, being capable of detecting on the order of 250 single strand breaks and/or alkali-labile sites in the DNA of a single cell (Singh et al., 1988). While not all DNA lesions are alkali-labile (neither are all lesions repaired by a long-patch repair process, nor do all lesions result in visible cytogenetic damage), many classes of lesions are labile under alkaline conditions (Swenberg et al., 1976; Yang et al., 1984). Furthermore, since this technique can be used to follow the excision repair of alkali-insensitive lesions, as demonstrated by the presence of single strand breaks formed during the repair process, the absence of alkali-labile sites may not be a critical problem.

It is this SCG technique that has been evaluated for use as a primary approach for detecting the possible exposure of mammalian organisms to genotoxic pollutants. In this final, two-year report, the experiments conducted to develop and characterize the assay and data obtained from studies to explore the sensitivity of the assay for detecting genotoxic damage induced in vitro and in vivo are presented. In many of these experiments, specific attention has been paid to the ability of acrylamide, dimethylbenzanthracene and trichloroethylene, three representative environmental pollutants, to induce single strand DNA breaks and/or alkali-labile sites in the DNA of mammalian cells. The principal purpose of this contract has been to expand the application of the SCG assay to the detection of DNA damage induced by chemicals in mammalian cells in vitro and in vivo and ultimately to the assessment of genotoxic damage in resident free-living animals or in humans environmentally exposed to hazardous pollutants. Data obtained should help to better characterize environments that pose a significant health hazard to mammalian species, including man.

2.0 THE BASIC SCG TECHNIQUE

Up to 10,000 cells of a cell suspension are mixed with 75 ul of 0.5% low melting point agarose at 37°C and then placed on a precleaned, fully-frosted microscope slide previously coated with 0.5% regular agarose (90 ul). The cell suspension is immediately covered with a #1 coverglass (40 x 50 mm) and the slides kept at 4°C for 5 minutes to allow solidification of the agarose. After adding a third layer of low melting agarose, and allowing for solidification, the slides are immersed in a lysing solution (1% sodium sarcosinate, 2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, pH 10, 10% DMSO, and 1% Triton X-100, added fresh) at 4°C for 1 hour to lyse the cells. The slides are then removed from the lysing solution and placed on a horizontal gel electrophoresis unit. The unit is filled with fresh electrophoretic buffer (1 mM Na_2EDTA and 300 mM NaOH; pH >13) to a level 0.25 cm above the slides. The slides are allowed to set in this high pH buffer for 20 minutes to allow unwinding of the DNA followed by electrophoresis for 10 to 40 minutes at 25 volts. All of the steps described above are conducted under yellow light or in the dark to prevent additional DNA damage. After electrophoresis, the slides are rinsed gently, to remove alkali and detergents which would interfere with ethidium bromide staining, by flooding them slowly with 0.4 M Tris, pH 7.5. After three 5 minute rinses, the slides are stained by placing 50-75 ul of a 10 ug/ml ethidium bromide solution in distilled water on each slide and covering the slide with a coverglass. Observations are made using a Zeiss fluorescent microscope equipped with an excitation filter of 515-560 nm and a barrier filter of 590 nm. This protocol is schematically presented in Figure 1.

3.0 IMAGE ANALYSIS SYSTEM

In the studies published by Singh et al. (1988), DNA migration patterns were determined by photographing representative cells on the gel, developing the negatives and measuring DNA migration length on a small number of cells using 10x magnifying device with an inset millimeter ruler. Not only is this method extremely labor intensive, the resulting data are of limited accuracy for detecting small but biologically important differences in migration. These two facts led us to seek an alternative method for obtaining DNA migration data. After reviewing a number of direct and indirect systems, a microscope-based image analyzer was selected as the method offering the greatest sensitivity and efficiency. After comparing various image analyzing systems, the Cambridge Instrument's Quantimet 520 image analyzer was selected and placed into operation late in the winter of 1989. All of the data presented in this report originate from this imaging device.

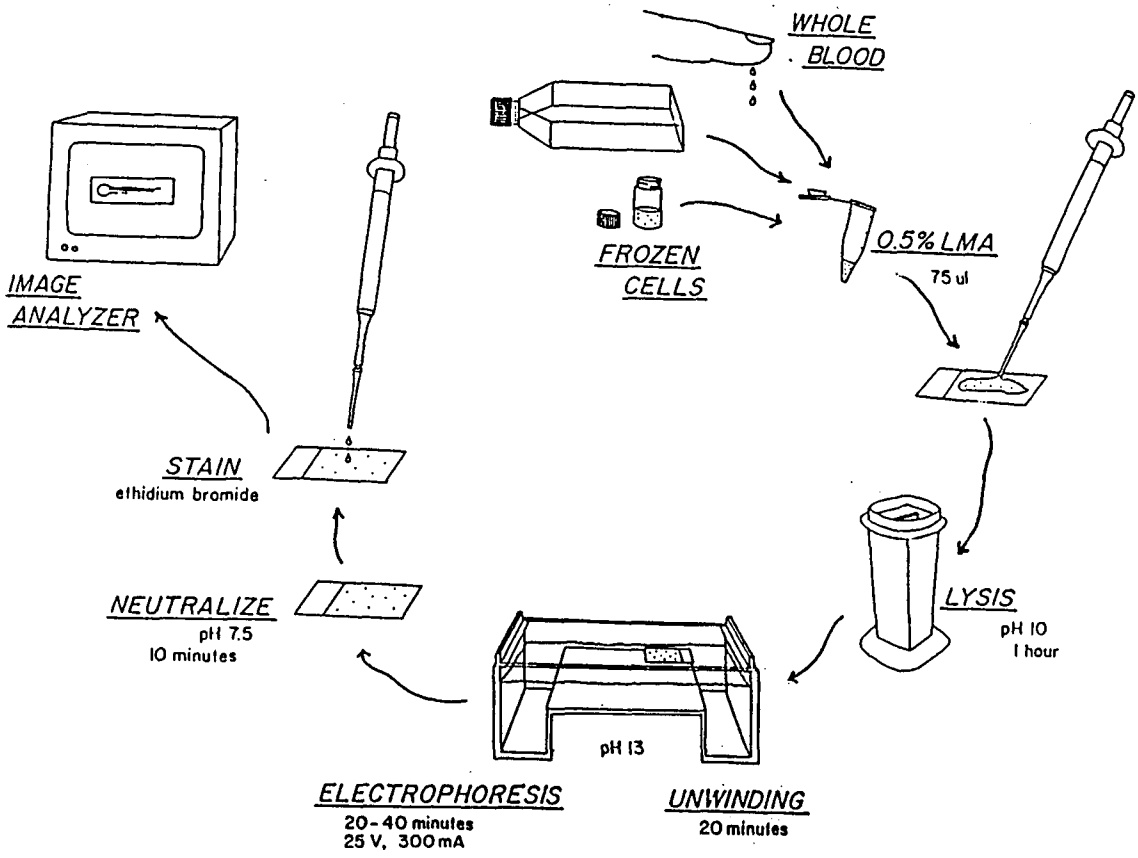

Figure 1. Schematic Presentation of the Technical Steps Involved in the Singel Cell Gel (SCG) Assay.

The Quantimet 520 consists of a gated CCD camera attached to the fluorescent microscope and wired into the image analysis hardware. The hardware is in turn attached to a graphics monitor for visualization of the digitized image, a mouse-controlled digitablet for editing the image, a dot matrix printer, and a Zenith 386 PC with a separate graphics monitor for running the Cambridge software. The Cambridge software allows for the setting of brightness and contrast levels, saving the image in memory, setting image intensity detections thresholds, editing and/or amending the image, calibrating to relative units, and finally measuring the migration length electronically. This process, although far better than that employed earlier, was still time consuming. To streamline the cell measurement process, a program in QBASIC (a Cambridge modification of the BASIC programming language) was written. The first version of the program, which still required manual manipulation of the software, could only measure the migration length in screen pixels and give a rough estimate of DNA intensity. The current program (Figure 2), which is in its ninth version, first sets optical shading, then asks for a data file name to store results. From the main menu, the user may input what objective is being used, the type of cell being scored (e.g., parenchymal, testis, lymphocyte), how many cells will be scored per slide, the size of the measuring frame, and pixel detection thresholds. When finished, calibration factors are automatically set and the current slide I.D. is entered. Once in the cell to cell loop, the scorer simply selects a cell, focuses it on the image monitor and presses the space bar. The program then stores the image in memory to reduce background and proceeds to first dilate (5 times) and then erode (5 times) the image. This step is necessary since many of the DNA migration tails are long, thin and spotty, and a continuous image is required for length measurement. An EDIT screen gives the scorer the option of removing unwanted background objects and/or connecting large tail pieces to the nucleus. The parameters finally recorded are the cell count, migration length (now calibrated in microns), a shape factor ($perimeter^2/area$), the diameter of the nucleus (also in microns), and the intensity of the migration tail. Tail intensity allows an approximate quantitation of the amount of DNA that has migrated from the nucleus. Thus, even in the absence of any DNA movement in the gel system, all cells have a minimal image length (called here DNA migration), a shape close to 1.0 (i.e., round), a minimal diameter and a measurable tail intensity. It should be noted that while shape is dimensionless and thus independent of call type, all of the other measurements have characteristic values for the type of cell being evaluated for DNA damage (e.g. parenchymal vs. nonparenchymal cells, diploid testes vs. haploid testes cells). These data are stored in a DOS file which can later be accessed for adding more information. After scoring the proper number of cells per slide, the program loops back to the main menu for the next slide. With this program, scoring time has been reduced to approximately 30 seconds per

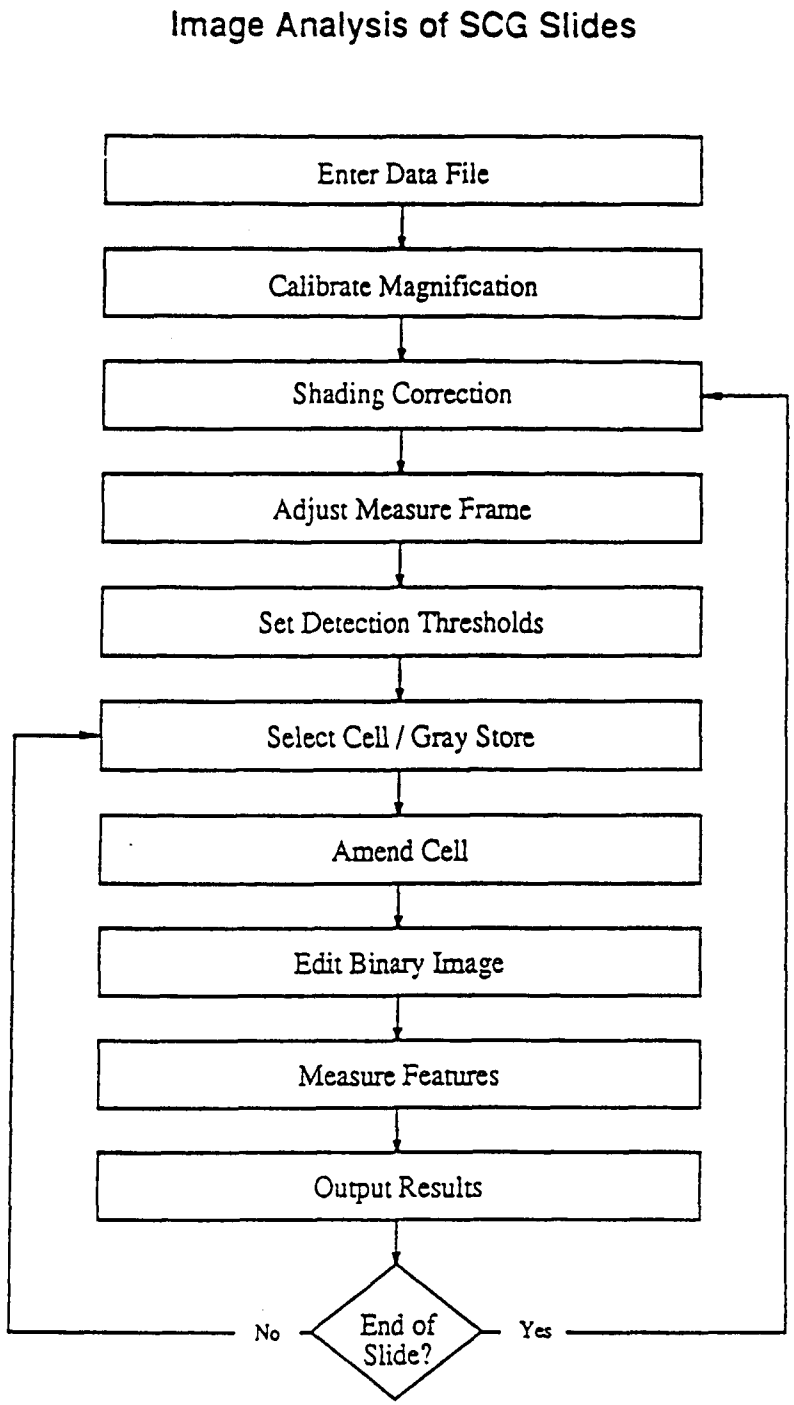

Figure 2. Flow Chart for the SCE Image Analysis Process.

cell (10-15 minutes per slide if 25 cells are scored). A spreadsheet template in QUATTRO PRO (software similar to Lotus 1-2-3) has been prepared which imports the data file, calculates means and standard errors for each slide, evaluates frequency distributions, then presents the data in tabular form. Sigmaplot, a graphics software package, is used to create the line and bar graphs used for presentations.

4.0 TECHNICAL PROBLEMS

In developing an image analysis-based SCG system and in applying the technique to different biological systems, a number of technical parameters had to be evaluated. In addition, problems throughout the course of the project were encountered which had to be solved.

4.1 Fluorescence Exposure Duration

The first parameter evaluated was the effect of the duration of exposure to the incident fluorescent light on the magnitude of the emitted ethidium bromide fluorescence signal from the image. In this experiment, the length of DNA migration for a cell with a short migration pattern and for a cell with an extended migration pattern was repeatedly measured (25 times over a 15 minute interval). The cells used in this experiment were human peripheral blood leukocytes randomly selected on a slide electrophoresed for either 10 or 20 minutes. The resulting data demonstrated initial rapid quenching of the fluorescent signal over the first 5 minutes, followed by a slower decline in signal strength over the remaining 10 minutes. This experiment indicated the necessity of measuring only one cell within the same microscope field of view.

4.2 Slide Placement

The second problem formally evaluated was the effect of slide placement (on the horizontal gel electrophoresis unit) on the resulting DNA migration patterns. In the first experiment, human blood cells were mixed with agarose and placed on several microscope slides. After lysis, a slide was placed on the horizontal gel box (18 x 24 cm) at each of the four corners and then electrophoresed for either 20 or 40 minutes. An analysis of DNA migration patterns among 25 cells selected in the same area on each slide revealed that the more distant the slide was from the anode, the less the average migration (Figure 3). The differential effect was more pronounced the greater the extent of DNA migration present among the cell population being scored. A second experiment resulted in a similar finding, while a third experiment (Figure 4) using a larger gel box (26 x 37 cm) revealed less of a position effect for the same average migration length. These data indicate the need to be aware of the potential for bias in migration resulting from slide placement and that more homogeneous data are obtained when slides are

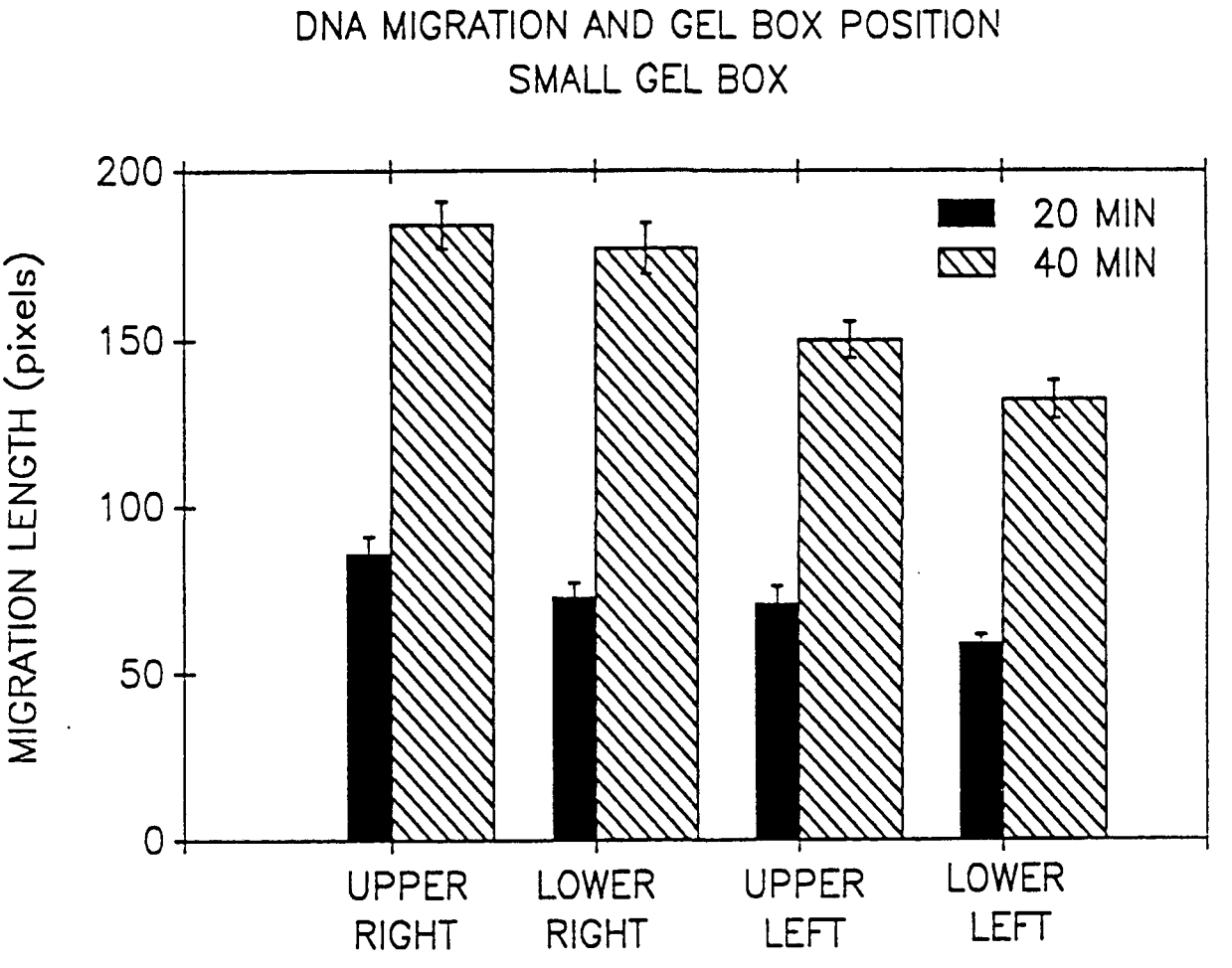

Figure 3. Slide Position on Small Gel Box and DNA Migration Length. Group mean data are presented, error bars indicate standard error of the mean among cells. The indicated positions are in relation to the anode, which is attached to the upper right. Data based on 25 cells scored per slide.

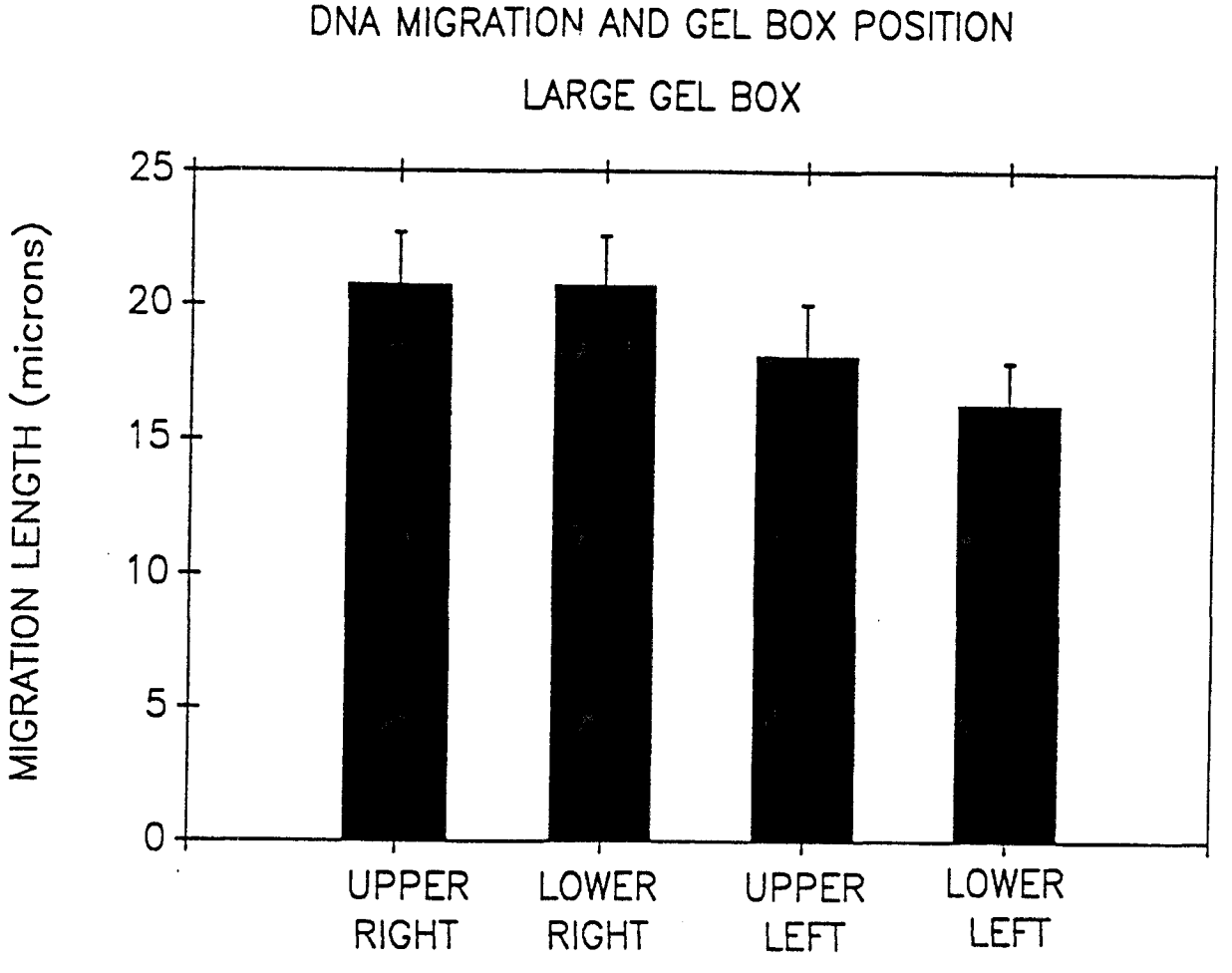

Figure 4. Slide Position on Large Gel Box and DNA Migration Length. Group mean data are presented, error bars indicate standard error of the mean among cells. The indicated positions are in relation to the anode, which is attached to the upper right. Data based on 25 cells scored per slide. Electrophoresis duration was 20 minutes.

electrophoresed on a larger gel box. Currently, to minimize this effect, replicate slides are randomly dispersed on the gel box, with at least one control slide placed closest to the anode.

4.3 Cell Position on the Slide

To evaluate the possible effect on DNA migration of the position of a cell on an individual slide, microscope slides were arbitrarily divided into thirds and 15 cells in each third were evaluated for DNA migration patterns. Slides electrophoresed for 20 and 40 minutes were used so that we could evaluate the effect of small or large migration lengths. In each case, cells were selected for scoring by moving in a preset number of viewing fields from the top edge to the bottom edge of the slide. This pattern would also enable an analysis of across slide position effects. While cell to cell variability in DNA migration lengths were present, no position-related differences in DNA migration were detected (Figure 5).

4.4 Electrophoresis Duration

In one of the first series of pilot electrophoresis experiments, human and mouse peripheral blood leukocytes were electrophoresed over a range of times (5 to 25 minutes) to evaluate optimal electrophoresis durations. Representative data from one experiment is presented in Figure 6. These initial studies indicated technical problems in conducting the assay since the DNA migration lengths exhibited by cells electrophoresed for 20 minutes were much greater than anticipated from data collected in N.P. Singh's laboratory (see Singh et al., 1988). Furthermore, the extent of DNA migration increased dramatically when the slides were stored in the lysis solution for longer than a few hours. This observation was also inconsistent with data from N.P. Singh, who observed no change in migration patterns among cells stored in the lysis solution for at least a week. Initially, considerable effort was spent in attempting to determine the source of this problem, evaluating such factors as the source of water used in solution preparation, the company from which the reagents were purchased, the types of containers used in the experiments, and other experimental conditions. None of these factors were responsible for the high level of DNA damage observed among control cells. Ultimately, much of the difficulty was traced to the formation of iron-mediated free radicals generated during lysis (see below).

4.5 Radical Induced DNA Damage

As mentioned above, it became apparent in our earlier experiments that, under apparently identical experimental condition, the processing of whole blood resulted in leukocytes with greater DNA migration than that observed for cells isolated from blood by Ficoll-hypaque centrifugation. The observation that the lysing

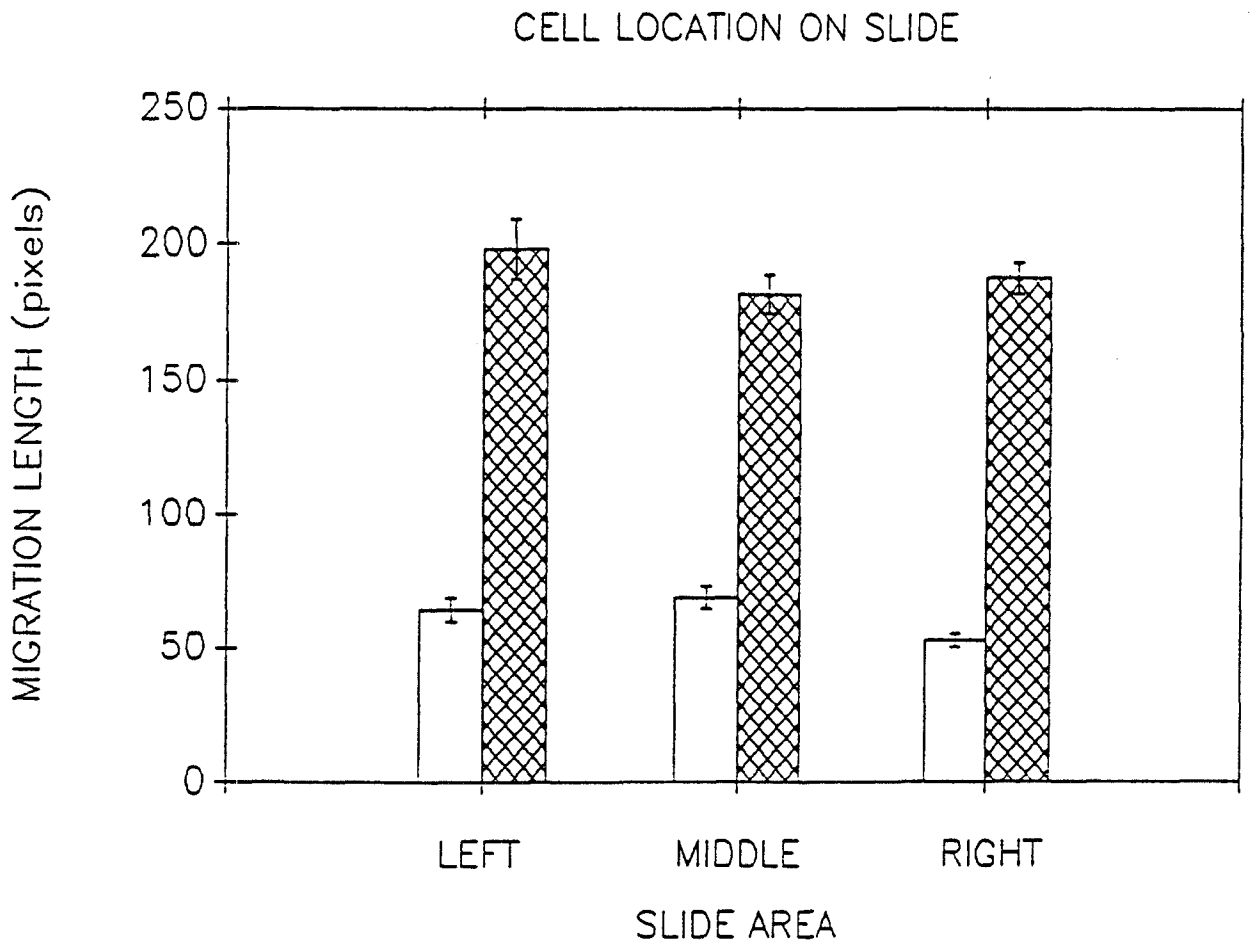

Figure 5. cell Position on a Slide and DNA Migration. A slide was divided into right, middle and left sections and 15 cells were scored in each section, while scanning from top to bottom of the slide. Group mean data are presented, error bars indicate standard error of the mean among cells. Open bars are for slides electrophoresed for 20 minutes, hatched bars for slides electrophoresed for 40 minutes.

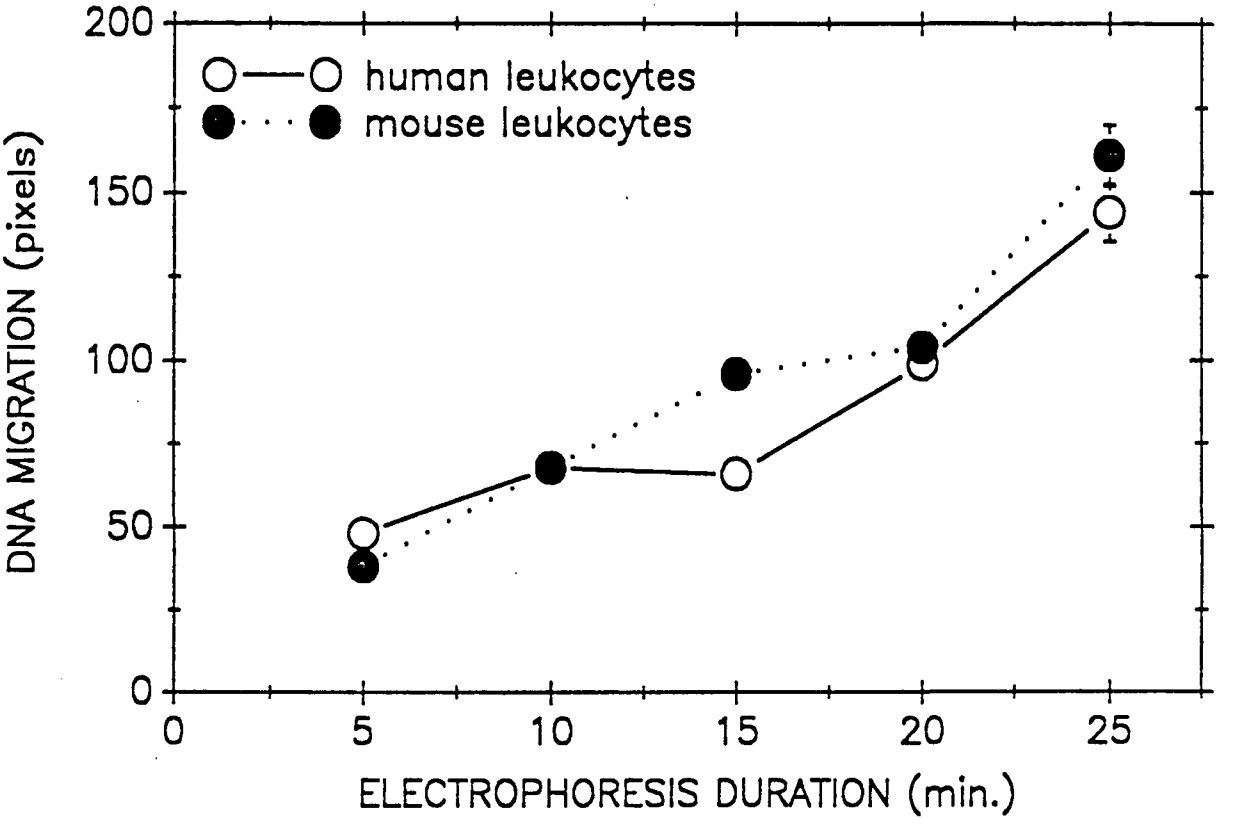

Figure 6. Electrophoresis and DNA Migration for Human and Mouse Leukocytes Processed as Whole Blood. Data are group menas for 25 cells, error bars indicate the standard error of the mean among cells.

solution turned pinkish when whole blood was lysed, suggested the presence of liberated hemoglobin and/or iron. Additionally, during this time, considerable inter-experiment variability in the extent of DNA migration among cell samples from control animals was occurring. It occurred to us that iron-mediated free radical generation may be responsible for the damage induced during lysis and for the excessive interexperiment variability. To evaluate this possibility, phenanthroline and desferroxamine, two iron chelators, were tested for their ability to suppress the increased level of DNA damage in leukocytes lysed as whole blood. In the first experiment, phenanthroline, dissolved in dimethylsulfoxide (DMSO) due to its insolubility in water, was tested over a range of concentrations. While the resulting data demonstrated the complete lack of a phenanthroline inhibitory response (in fact the compound appeared to induce DNA damage), it appeared that DMSO alone was capable of inhibiting the increased levels of DNA damage induced during the lysis step (Figure 7). Two additional experiments with DMSO were subsequently conducted. First, increasing concentrations of DMSO were evaluated for their ability to inhibit the induction of DNA damage during lysis and 10% DMSO was determined to be the optimal concentration. Second, it was determined that the presence of DMSO had no effect on DNA damage induced in mouse leukocytes by hydrogen peroxide prior to the lysis step. Furthermore, DMSO appears to protect cells in lysis from damage for at least two weeks. Presently, DMSO is routinely included in the lysis solution.

Incubation of whole blood slides in desferroxamine also inhibited the induction of DNA damage in human leukocytes during lysis (Figure 8). Because the sample of desferroxamine available was small, a complete range of concentrations could not be tested. Since this compound is a specific chelator of free iron, these data suggest the importance of eliminating iron from the lysis solution.

4.6 Cell Fixation

An extensive series of experiments were conducted to evaluate various methods of cell fixation prior to SCG analysis. We hoped that a method could be developed whereby cell samples could be stored and then processed when convenient for the SCG assay. This step would greatly increase the utility of the assay by delaying the need for immediate laboratory processing. A number of fixation methods were attempted, including fixation in absolute methanol, in 70% methanol, in 3:1 methanol:glacial acetic acid, in glacial acetic acid, and in various concentrations of glutaraldehyde. All of these methods resulted in DNA which, when processed using the usual SCG technique, was overly condensed when stained and imaged on the gel. This state of contraction indicated a lack of normal unwinding during lysis and an inability to undergo normal movement during

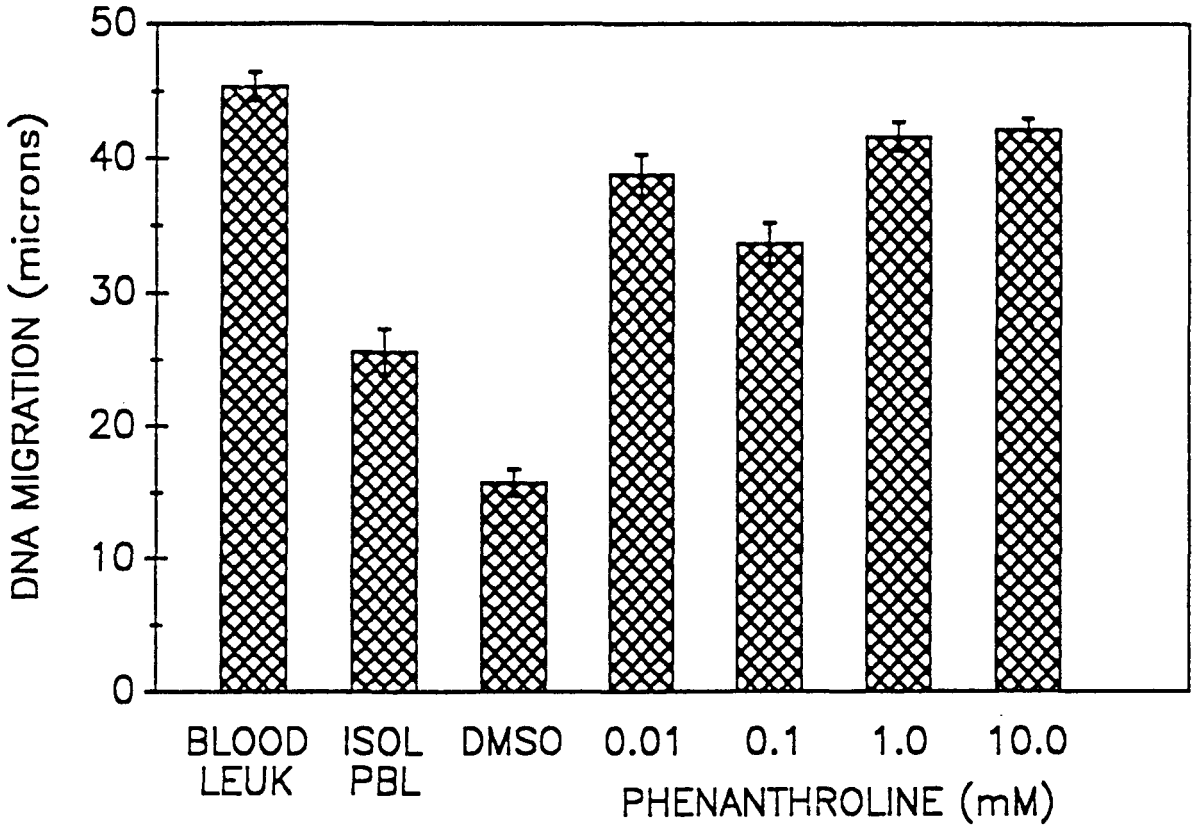

Figure 7. Effect of DMSO (10%) and Phenanthroline on DNA Damage Induced in untreated Mouse Leukocytes during Lysis. Data are group means for 25 cells, error bars indicate the standard error of the mean among cells. LEUK = leukocytes; ISOL PBL = Ficoll-isolated peripheral blood lymphocytes.

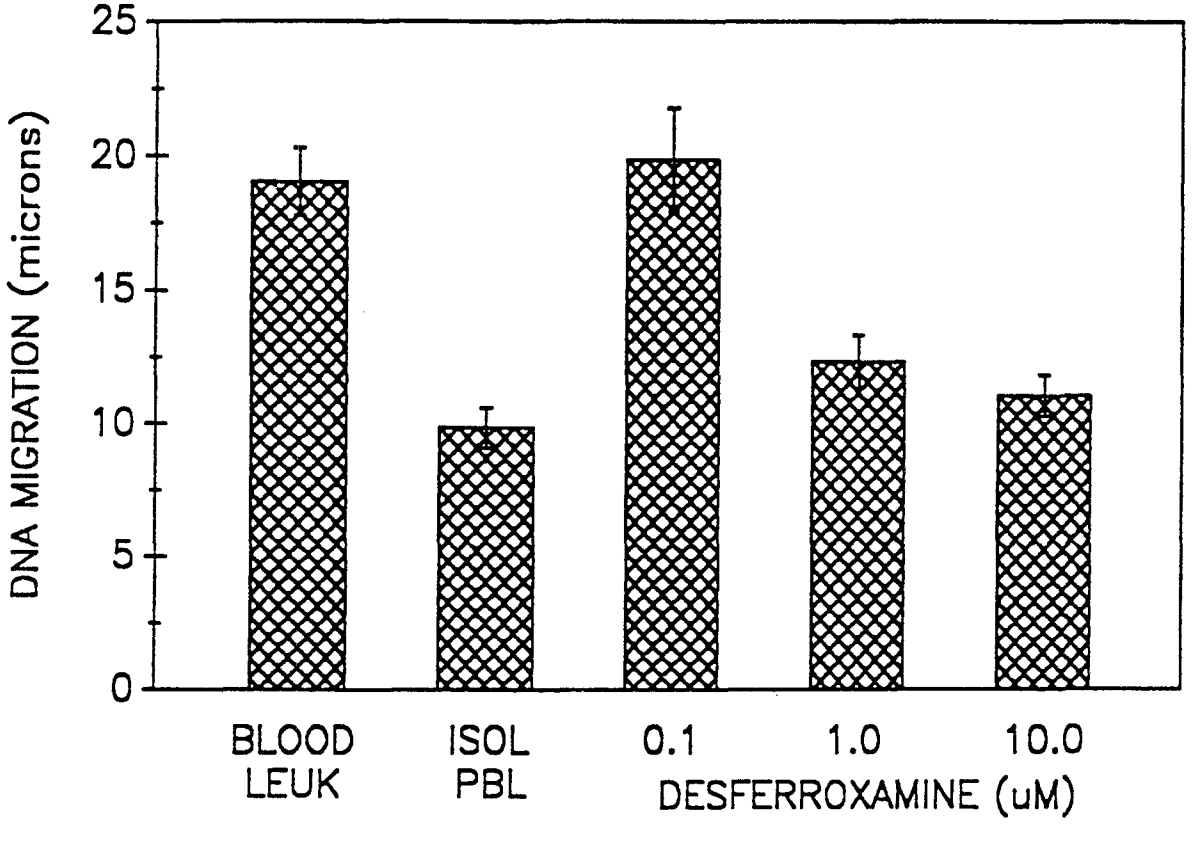

Figure 8. Effect of Desferroxamine on DNA Damage Induced During Lysis of Mouse Whole Blood. Data are group means for 25 untreated control cells, error bars indicate the standard error of the mean among cells. LEUK = leukocytes; ISOL PBL = Ficoll-isolated peripheral blood lymphocytes.

electrophoresis. While experiments to evaluate other methods or these same methods under different experimental conditions have not been conducted, the potential utility of a proper fixation method suggests the importance of additional studies at some time in the future.

4.7 Processing Delays

As more complex experiments were undertaken, it became necessary to identify steps in the assay which could be delayed without compromising the integrity of the experiment. As already discussed, it proved possible to leave the cells in lysis for at least a few weeks once DMSO was routinely incorporated into the lysis solution. The only other steps considered to be amenable to processing delays were at the time of cell collection or after the cells had been stained with ethidium bromide, but before image analysis. Since a delay in sample processing prior to lysis was not considered initially to be a problem, our first efforts focused on evaluating how long stained slides could be retained before scoring. In these experiments, the DNA migration patterns for a randomly selected cell population (generally mouse or human peripheral blood leukocytes) were determined immediately after staining or after storage of the slides. Initially, we determined that slides stored in a cold, humidified, sealable container could be accurately scored over a period of 3 to 4 days. Longer storage durations resulted in the excessive diffusion of small pieces of DNA away from the tail. The maximum length of storage time is dependent on the initial size of the image being analyzed. DNA diffusion from smaller cells (e.g., spermatocytes) created a greater problem over the same period of time than DNA diffusion from larger cells (e.g. parenchymal cells). To evaluate the possibility of storing the slides for more extended periods, some gels were dehydrated in 70% or 100% methanol and stored cold. Subsequent rehydration, with or without additional ethidium bromide staining failed to provide usable fluorescence images. We also evaluated whether freezing gels at -20°C or -70°C could be used for long term slide storage. Best results were obtained with quick -70°C freezing and slow thawing. However, this process also resulted in agarose dehydration and increased background from the slide frosting.

4.8 Dead Cells

Our major concern about the SCG assay, as would be for any assay based on the detection of single strand breaks, is the possible effect of the presence of dead cells on data analysis. In conducting a number of SCG experiments, it was generally noted that some cells on the gel exhibited no point of origin (i.e., no originating cell nucleus remained, but rather a cloud of migrated DNA). Based on a significant correlation between their frequency and viability data, we concluded that these objects represented

dead cells. To determine the accuracy of this conclusion, human peripheral blood leukocytes were isolated by Ficoll-hypaque centrifugation, and then incubated for 60 minutes at 60°C. Trypan blue exclusion was used to indicate viability. Under conditions which resulted in 100% dead cells, all cells appeared as clouds. Our thought was that any dead cell would exhibit this same pattern of migration. However, data obtained on cells exposed to 60°C for a shorter time suggested a continuation of DNA migration patterns. A comparison with vital dye exclusion frequencies suggested that the cloud pattern depended on how long the dead cell had been dead (i.e., the autolysis of DNA to extremely small fragments takes time). These data suggest the need to routinely obtain viability data and to conduct experiments at doses that do not result in a significant depression in viability (i.e., below 80-90%). More recently, we have been using a dual viability stain consisting of 5-6 carboxyfluorescein diacetate (CFDA) and ethidium bromide. In this technique, ethidium bromide stains the DNA red in any cell with a compromised cell membrane, while CFDA stains the cytoplasm green in any cell metabolically active. Thus, the combination of the two stains permits an assessment of dead cells (red with no green), compromised cells (red and green) and live cells (green but not red). Currently, as a quality assurance check, cells are routinely monitored for viability prior to SCG analysis.

4.9 Cell Selection Criteria

One of the major potential sources for bias in this assay is the basis on which individual cells are selected for analysis. Since the extent of intercellular DNA damage is very heterogeneous in many cases, it is quite possible that a scorer may be biased towards selecting cells with more or less damage. This is especially true if the cell density on the slide is high (i.e., more than 2-3 cells per field). To reduce this possibility, a set criteria for cell selection was developed. In scoring a slide, the scorer scans the slide either horizontally or vertically, limiting image analysis to cells which intersect the image box on the computer monitor. Using this approach, sample bias is largely avoided. To demonstrate the success of this approach, we have determined for a number of control and treated slides the distribution of cells with no tails, short tails, long tails, and with no origin (i.e., clouds) among 100 cells and compared the results with the distribution of cells among the various cell types actually analyzed for DNA migration. The differences have been insignificant.

4.10 Other Technical Problems

Several factors were found to be critical in maintaining proper background control levels and gel quality on the slides. At one point, a defective batch of fully frosted slides was

received from the supplier. These slides apparently were not ground as well as previous batches. This resulted in a significant proportion of the agarose gels sliding off the slides during processing (with the complete loss of several experiments). We determined that this problem could be circumvented by increasing the agarose concentration of the bottom layer to 0.6%. Currently, when a new batch of microscope slides is obtained, the ability of the standard first agarose layer to remain attached to a number of slides is checked.

Another critical factor was the pH's of the lysing solution and the electrophoresis buffer. During the normal shelf life of these solutions, it was discovered that the pH can shift by as much as one pH point in either direction. An increased pH in the lysing solution results in increased migration tails, while a decreased pH in the electrophoresis buffer results in the lack of DNA migration. The pH of both these solutions are now routinely checked and adjusted prior to use.

Yet another problem occurred when new Coplin jars were purchased. Several of these jars contained a residue which resulted during the lysis step in increased DNA migration tails. Extensive cleaning of these coplin jars with DMSO ended this problem.

A fourth problem was the frequent high background fluorescence from the slide frosting which interfered with slide scoring. This problem was corrected by simply increasing the volume of the bottom agarose layer from 75 to 95 microliters and by decreasing the volume of ethidium bromide from 75 to 50 ul.

Lastly, it was discovered that stock bottles of Triton X-100, one of the detergents in the lysing solution, are extremely prone to bacterial growth when opened frequently. This also resulted in longer migration tails amongst our controls. This problem was solved by aliquoting 10 ml samples into smaller bottles for daily use.

5.0 IN VITRO EXPERIMENTS

A series of in vitro studies were conducted to investigate the applicability of the SCG assay to the detection of chemically-induced DNA damage in mammalian cells treated in vitro with a variety of DNA damaging agents.

5.1 S9 Mix and DNA Migration

To investigate in vitro the genotoxic potential of chemicals, an exogenous source of metabolic activation is often provided to metabolize inert parent compounds to reactive species. This is

normally accomplished by adding an S9 mix, a source of mixed function oxidases obtained from the liver of rats (generally Aroclor-treated). In this study, isolated mouse blood leukocytes were incubated in complete medium with and without the addition of an S9 mix at 37°C for 1 to 4 hours and the extent of DNA migration determined in cells electrophoresed for 15 minutes (Figure 9). Although the effect was small, the resulting DNA migration suggested an increase in DNA damage which depended both on the incubation time and the presence of rat liver S9.

5.2 Chemically Induced DNA Damage

5.2.1 Human Leukocytes: The first series of experiments involved the exposure of mammalian cells to hydrogen peroxide (H_2O_2). The SCG assay is an extremely sensitive technique for evaluating free-radical induced DNA damage, such as that caused by this reactive species. We decided to examine the differential ability of H_2O_2 to induce damage in the DNA of intact mammalian cells as compared to the DNA of cells after lysis. We considered the ability to test the DNA-damaging activity of chemicals against purified double-stranded DNA as one of the important aspects of the SCG technique. In this way, chemicals requiring intercellular metabolic processing could be discriminated from pure, direct acting chemicals. Five ul samples of whole blood were obtained from a single male donor, and added to 1 ml of RPMI-1640 containing 10% fetal calf serum. After centrifugation, the pellet was either suspended in Hanks calcium-magnesium free, balanced salt solution containing H_2O_2 (44 and 176 uM) for 1 hour at 4°C or mixed, as described, with 0.5% LMA and layered onto microscope slides. For the former method, the cells were collected by centrifugation, and processed for SCG analysis as described. For the latter, after 1 hour in the lysis solution, the slides were rinsed twice in cold PBS and then placed for 1 hour in a coplin jar containing H_2O_2 (44 and 176 uM) at 4°C. All control and treated slides were electrophoresed for 20 minutes, and 50 cells were scored per treatment.

At these concentrations, H_2O_2 induced a significant increase in the migration of DNA, regardless of whether metabolically active cells or lysed cells were treated (Figure 10). However, the mean extent of DNA migration appeared much greater for lysed cells than for intact cells. This apparent difference was due to the differences in the intercellular distribution of DNA damage between these two experimental conditions. When intact human leukocytes were exposed to H_2O_2, the intercellular distribution of DNA migration was much more heterogenous than that detected for lysed cells (Figure 11). Based on a dispersion analysis (where H, the dispersion coefficient, is equal to the variance/mean), the DNA migration lengths among the lysed cells exposed to H_2O_2 was distributed as a Poisson (i.e., randomly). Among the intact cell population, the majority of the cells

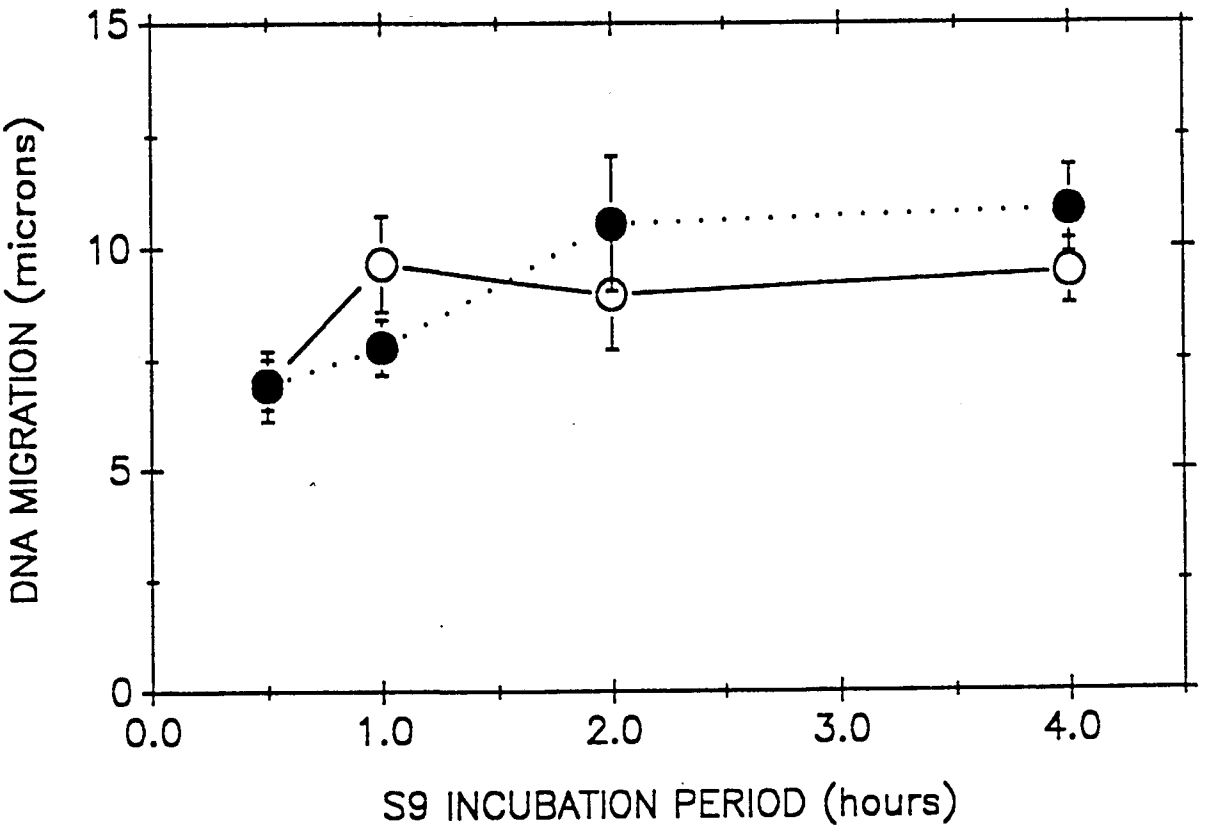

Figure 9. Effect of Incubation Duration and S9 Mix on the DNA Migration of Isolated Mouse Leukocytes. Data are group means for 25 cells, error bars indicate standard error of the mean among cells. Open symbols indicate cultures without S9, solid symbols indicate cultures with S9.

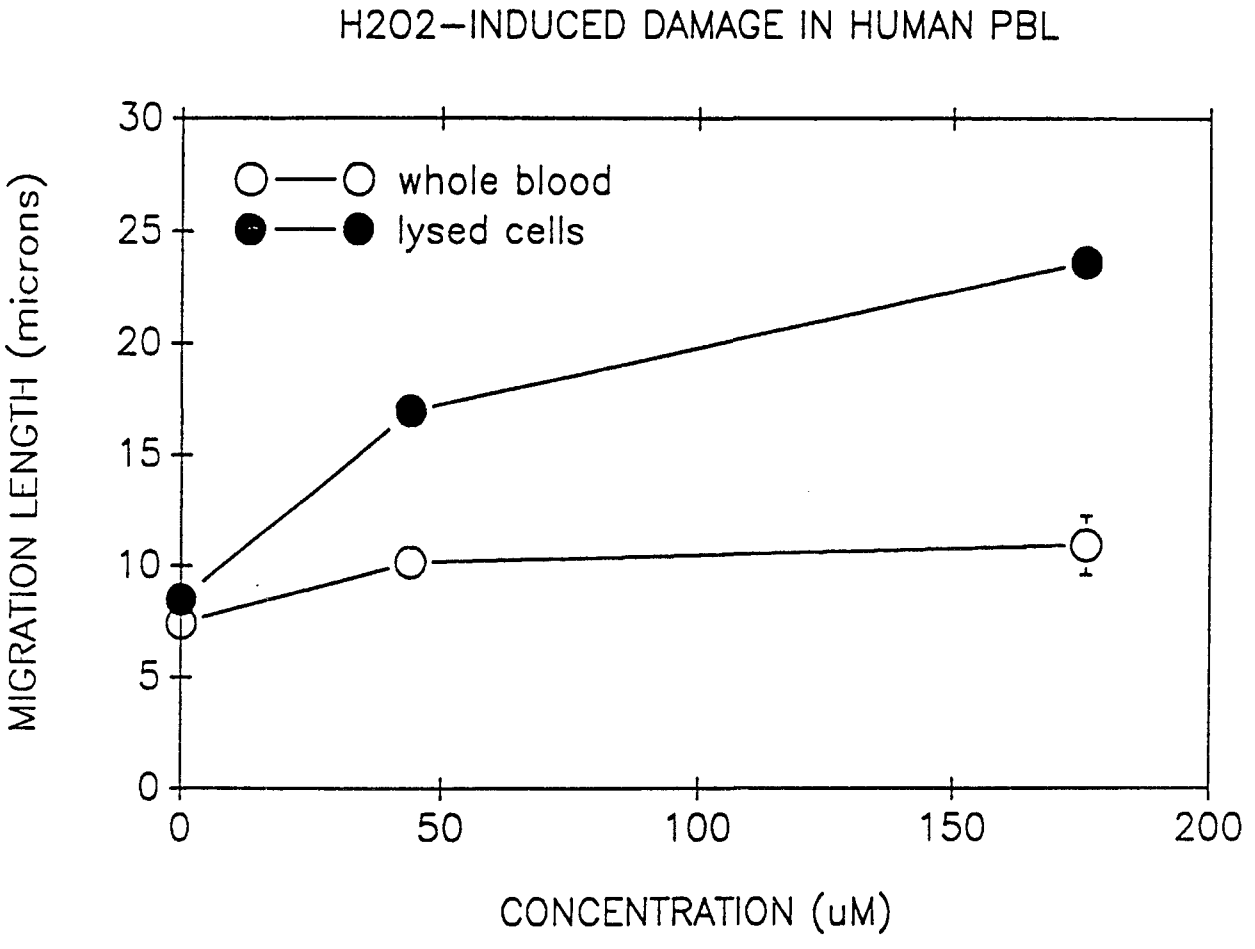

Figure 10. Mean DNA Migration for Intact Human Leukocytes or Lysed Cells Exposed to Hydrogen Peroxide. Data are group means for 50 cells, error bars indicate standard error of the mean among cells.

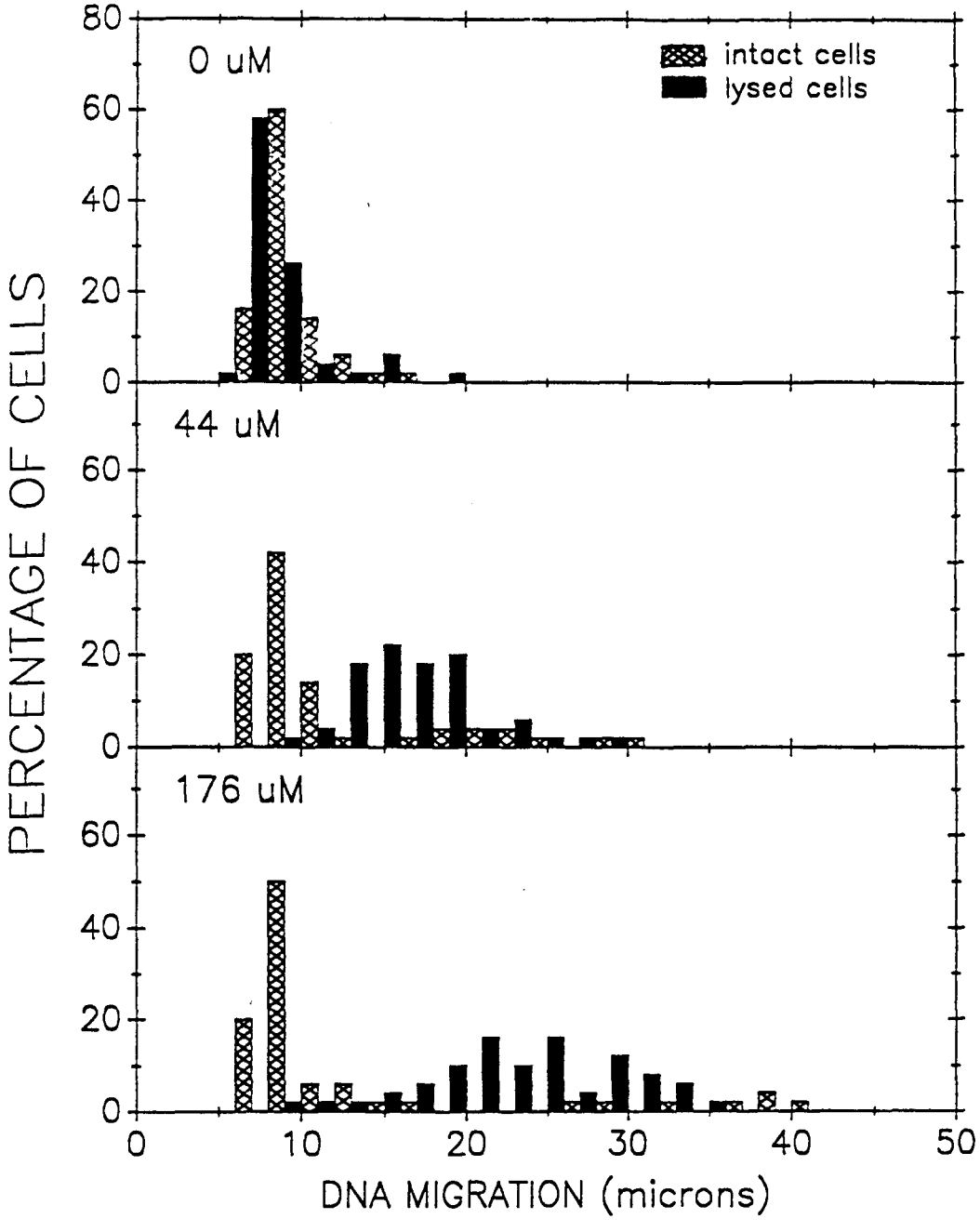

Figure 11. Intercellular Distribution of DNA Migration in Intact and Lysed Human Leukocytes Exposed to Hydrogen Peroxide. Data are for 50 cells per sample. The width of each bar represents 2 micron intervals.

exhibited no damage, with a subpopulation of cells expressing DNA migration patterns consistent with that detected among the lysed cells (Figure 11). These increased migration patterns could not be explained by the presence of dead cells among the exposed population since viability measurements routinely resulted in 97% to 100% viability. Several explanations are possible for this extensive heterogeneity in DNA damage among intact, metabolically active cells. For instance, individual cells may vary in their permeability to H_2O_2, their radical scavenging capabilities, the accessibility of DNA to the damaging species and other mechanisms which either enhance or diminish the effects of this potent radical.

5.2.2 Mouse Leukocytes: In the second series of in vitro experiments to formally evaluate the induction of DNA damage by a test chemical, ficoll-hypaque isolated mouse leukocytes were incubated with dimethylbenzanthracene (DMBA) at 1 to 50 ug/ml, in the presence of a standard rat liver S9 mix for 4 hours. DNA migration in treated cells appeared to be increased, but the increase did not depend on the dose of DMBA. Because the possibility existed that the S9 mix was not properly active, the next series of experiments used acrylamide (ACR), a direct acting chemical as the test agent. Isolated mouse leukocytes were incubated for four hours in the presence of ACR (5 to 1000 uM), including and excluding a rat liver S9 mix. DNA damage was not detected in either set of cultures. Subsequently, an experiment was conducted in which mouse blood leukocytes were incubated in complete medium at 37°C in the presence of ACR at 1000 uM for 5, 15, 30, 60 and 120 minutes. Exposures of short duration (30 minutes or less) resulted in a significant increase in DNA damage. By 1 to 2 hours, the extent of DNA migration was returning to control distances. These data indicate that the previous experiments were negative because the sample time was inappropriate, permitting sufficient time for DNA repair to remove the damage. Because DNA repair processes effectively repair the damage, we determined that the addition of cytosine arabinoside (ARA-C), a DNA synthesis chain terminator, could be used to prevent ligation of the repair sites during unscheduled DNA synthesis. Since, under these conditions, any DNA damage would either directly result in strand breaks and/or alkali-labile sites or indirectly result in single strand breaks formed as a consequence of DNA repair, this step makes it unnecessary to evaluate DNA damage induction kinetically. It would be of interest to more completely characterize this approach as it applies to routine testing.

5.2.3 Chinese Hamster Ovary Cells: To better standardize the in vitro SCG assay, we decided to focus additional research on Chinese hamster ovary (CHO) cells, a transformed fibroblast cell line commonly used in genetic toxicology. CHO cells were obtained from American Type Culture Collection, Rockville, MD and stored in liquid nitrogen. Stock cultures were maintained in

Ham's F-12 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 ug/ml streptomycin. All cultures were maintained at 37 $\pm$ 1°C in an atmosphere of 5 $\pm$ 1% CO_2 and 95% relative humidity. For the experiments, cultures were seeded 16-20 hours prior to treatment in 35 mm petri dishes at a density of 2 x 10^5 cells/dish. Dilutions of the 3 test chemicals were prepared in sterile phosphate buffered saline (pH 7.4) for ACR and DMSO for trichloroethylene (TCE) and DMBA. Based on data from experiments using primary rat hepatocytes (Hirai et. al., 1990), cyclophosphamide (CP), an alkylating agent requiring metabolic activation, was selected as a positive control for the S9 activated portions. Mitomycin C (MMC) and hydrogen peroxide, two direct acting compounds not requiring metabolic activation, were used as a positive controls for the nonactivated experiments. Exogenous metabolic action was provided by using a liver homogenate fraction (S9) prepared from Aroclor 1254-induced male Sprague Dawley rats. The final concentrations within the S9-cofactor pool were 4.5 mg isocitrate, 2.4 mg NADP and 0.02 ml S9 per milliliter of culture medium.

A preliminary toxicity test was performed to determine the optimum doses for testing in the SCG assay. Duplicate cultures seeded 16-20 hours earlier were exposed to five doses of each chemical, both with and without metabolic activation, in half log intervals starting with high doses of 10 mM for ACR and TCE, and 1 mM for DMBA. After 4 hours of exposure for the S9 activated cultures and 8 hours for the nonactivated cultures, cells were harvested by trypsinization and the percentage of viable cells calculated using the trypan blue dye exclusion method. Only doses with approximately 80% or greater viability were used in the SCG assay. For the DNA damage assay, overnight growth media was removed from the cultures and replaced with 2.0 ml of media with 5% FBS for the nonactivated cultures or with 1.6 ml media and 0.4 ml S9 mix for the S9 activated cultures. Twenty microliters of the test chemical dilutions were then added to the appropriate dishes in duplicate and all cultures placed at 37°C incubation. After 1-8 hour exposure periods for the nonactivated cultures and 0.5-4 hours for the S9 activated cultures, the test medium was removed, the cultures rinsed once in 1.0 ml cold Hank's Balanced Salt Solution (HBSS) and 0.5 ml more HBSS added. The cells were dislodged into the HBSS using a teflon scraper. Ten microliters of this cell suspension were mixed with 75 ul of LMA and layered onto microscope slides.

Acrylamide: Initial kinetic studies were performed with 10, 1.0 and 0 mM doses of ACR for 0.5, 1, 2 and 4 hours in the presence of S9 and for 2, 4, 6 and 8 hours in the absence of S9 activation. Increased exposure times resulted in an increase in the length of DNA migration (Figure 12). An earlier, significant increase over controls for ACR with S9 (2 hours vs 4 for the nonactivated cultures) was observed for both the 10 and 1.0 mM

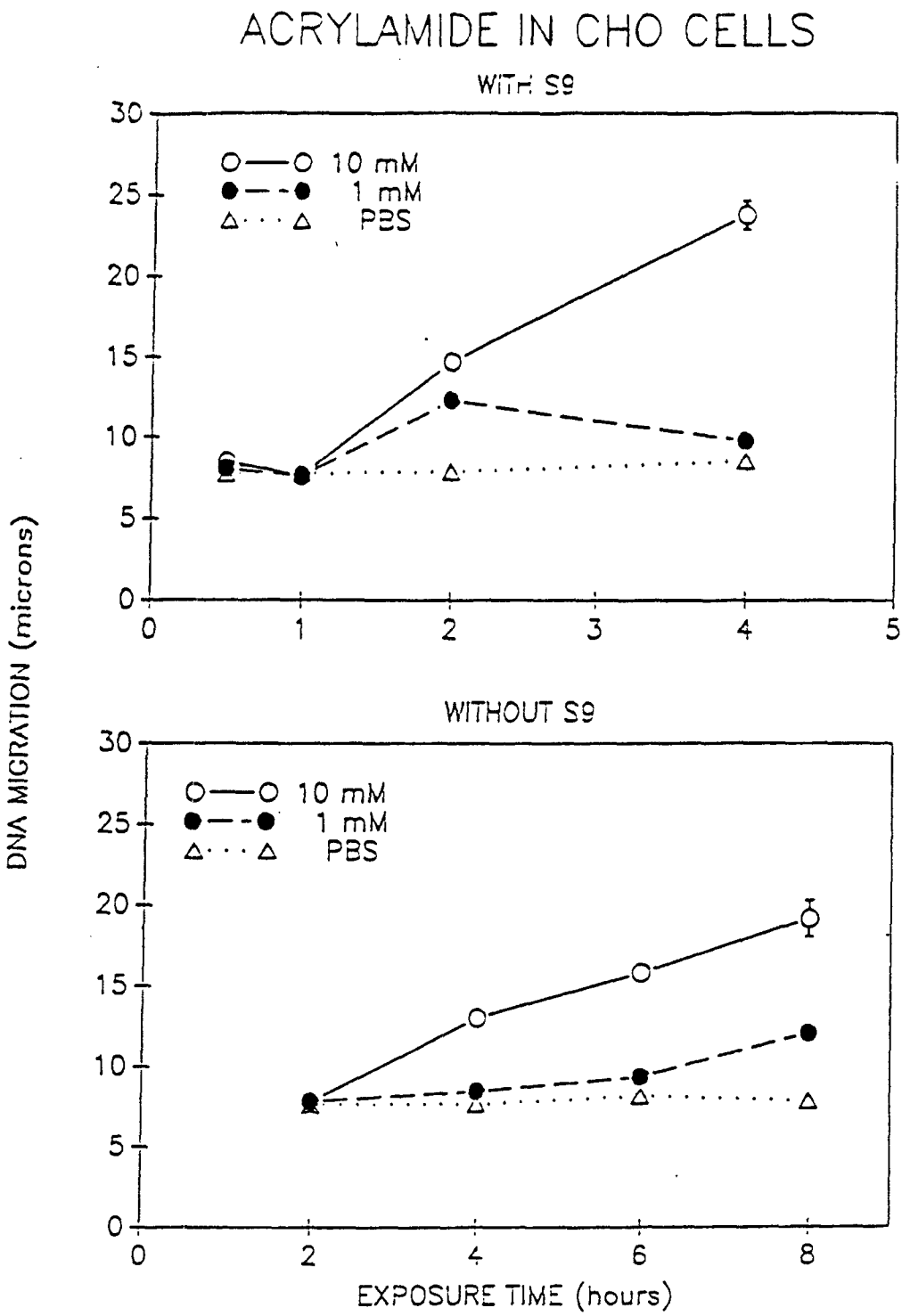

Figure 12. Mean DNA Migration for CHO Cells Exposed to Acrylamide as a Function of Sample Time in the Presence or Absence of Rat Liver S9 Mix. Each point represents the mean and standard error of the mean for duplicate cultures. For each culture 25 cells were scored.

doses. Control values (6-8 um) represent cell diameters after DNA unwinding only, with little to no DNA migration. Electrophoretic migration profiles for the DNA of cells after treatment with 10 mM ACR over time are shown in Figure 13 with S9 and Figure 14 without S9. These fragments represent a combination of frank acrylamide-induced breaks and breaks resulting from the hydrolysis of alkali-labile lesions under the conditions of the lysing and electrophoresis experiments. Based on these results, dose response experiments were conducted over a broader range of concentrations (0, 0.1, 0.5, 1.0, 5.0 and 10 mM) at a single exposure time (4 hours with S9 and 8 hours without S9). Increasing the concentration of ACR results in a progressive increase in the DNA migration length, indicating a dose-dependent generation of DNA strand breaks (Figure 15). At equal exposure times, ACR (10 mM) with S9 induced longer migration lengths than the same dose without S9 and twice the exposure time. Migration length frequency histograms for all doses are shown in Figure 16 with S9 and Figure 17 without S9.

Trichloroethylene: Identical kinetic studies were performed with 10, 1.0 and 0mM doses of TCE both with and without S9 activation. Increased exposure times resulted in an increase in the length of DNA migration for the S9 activated cultures only (Figure 18). In the absence of metabolic activation, a small but non-significant increase in the length of DNA migration was observed in cells exposed to 10 mM TCE after 6 hours of exposure. DNA migration profiles for CHO cells after treatment with the 10 mM dose over time with S9 are shown in Figure 19. Based on these results, a dose response experiment was conducted in the presence of S9 over a broader range of concentration (0.1-10 mM) at a single exposure time of 4 hours. Increasing the concentration of TCE likewise results in a progressive increase in the DNA migration length (Figure 20). A significant increase in the length of DNA migration over controls was observed in cells exposed to TCE at doses as low as 0.5 mM. The DNA migration histograms for the six doses are shown in Figure 21.

Dimethylbenz(a)anthracene: Exposure to 100, 10 and 0 uM of DMBA induced a time-dependent increase in DNA damage in the presence of metabolic activation only (Figure 22). After 2 hours of exposure, the level of DNA damage was significantly increased at the 100 uM dose relative to DMSO control levels. An analysis of the distribution of migration patterns among individual cells indicated a heterogenous response among cells in the top dose (Figure 23). No significant increase in the length of DNA migration was observed in cells exposed in the absence of metabolic activation even at the top dose and longest exposure time. A dose response experiment was conducted in the presence of S9 over a broader range of concentrations (1-100uM) at a single 4 hour exposure time. Increasing the concentration of DMBA resulted in a progressive increase in the DNA migration length (Figure 24). A significant increase in the length of DNA migration was observed

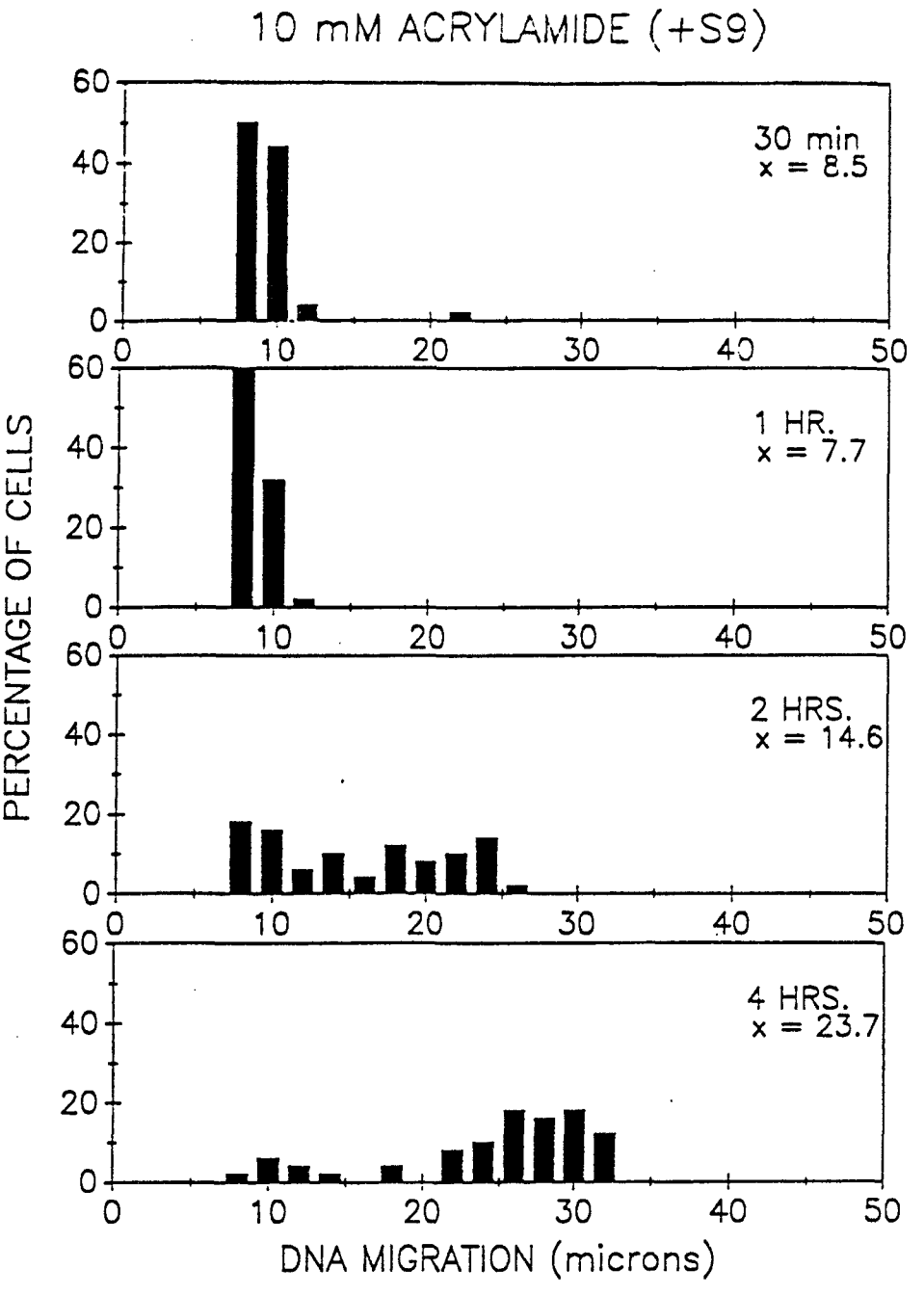

Figure 13. Sample Time Dependent Distribution of DNA Migration Patterns for CHO Cells Exposed to 10 mM Acrylamide in the Presence of Rat Liver S9 Mix. Data are for 50 cells per sample. The width of each bar represents 2 micron intervals. X = group mean DNA migration.

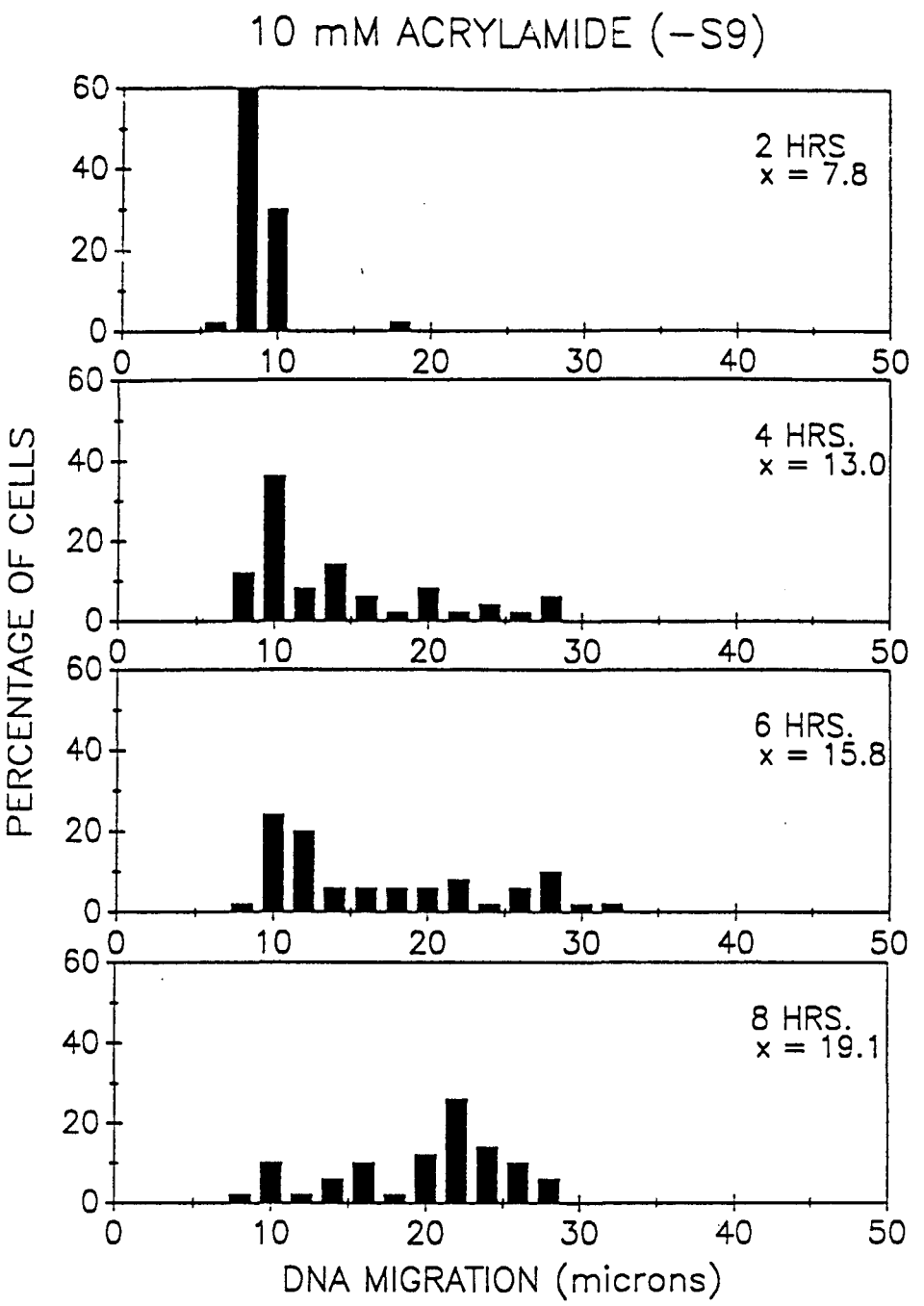

Figure 14. Sample Time Dependent Distribution of DNA Migration Patterns for CHO Cells Exposed to 10 mM Acrylamide in the Absence of Rat Liver S9 Mix. Data are for 50 cells per sample. The width of each bar represents 2 micron intervals. X = group mean DNA migration.

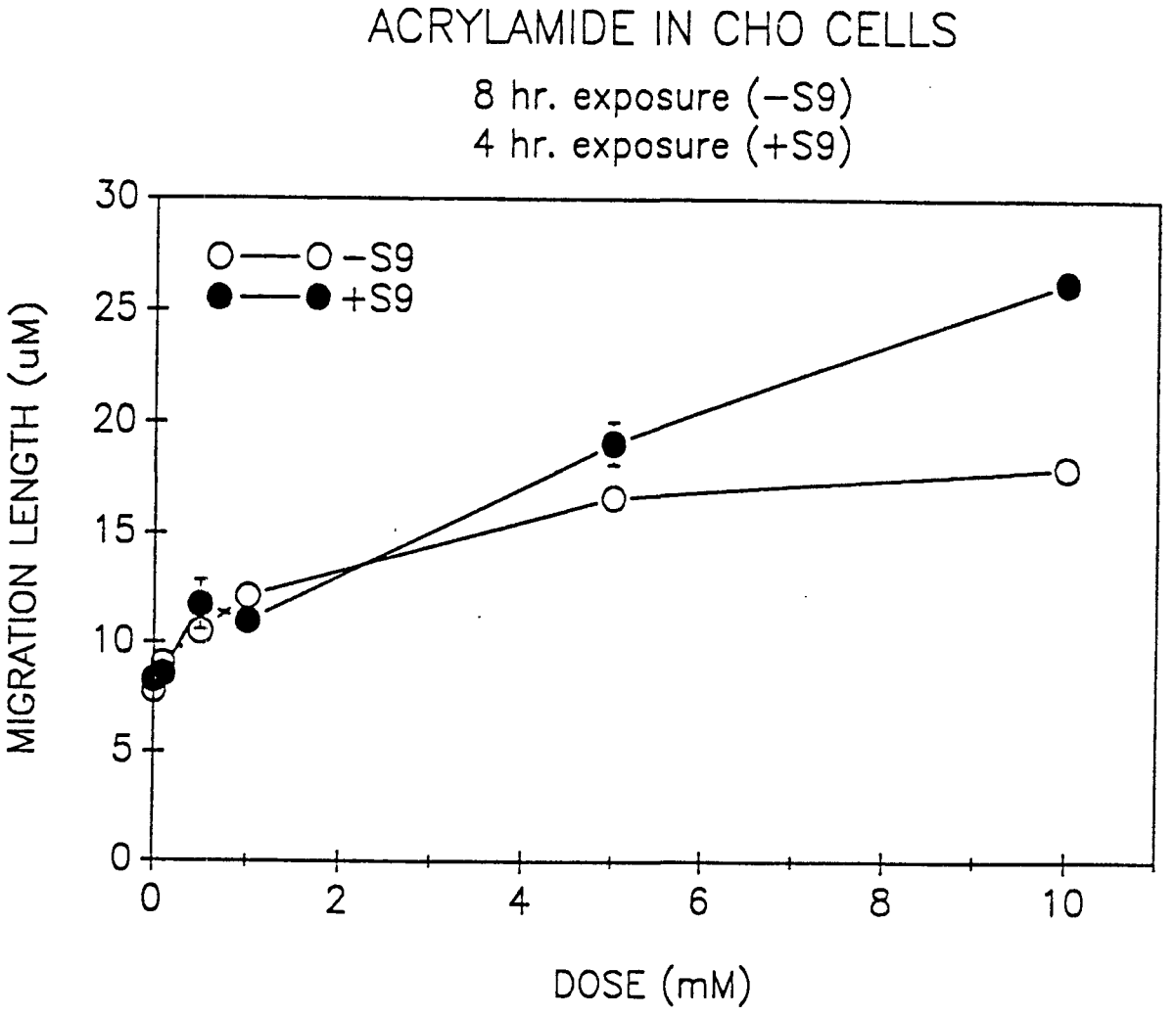

Figure 15. Mean DNA Migration for CHO Cells Exposed to Acrylamide as a Function of Dose in the Presence (4 Hour Treatment) or Absence (8 Hour Treatment) of Rat Liver S9 Mix. Each point represents the mean and standard error of the mean for duplicatie cultures. For each culture 25 cells were scored.

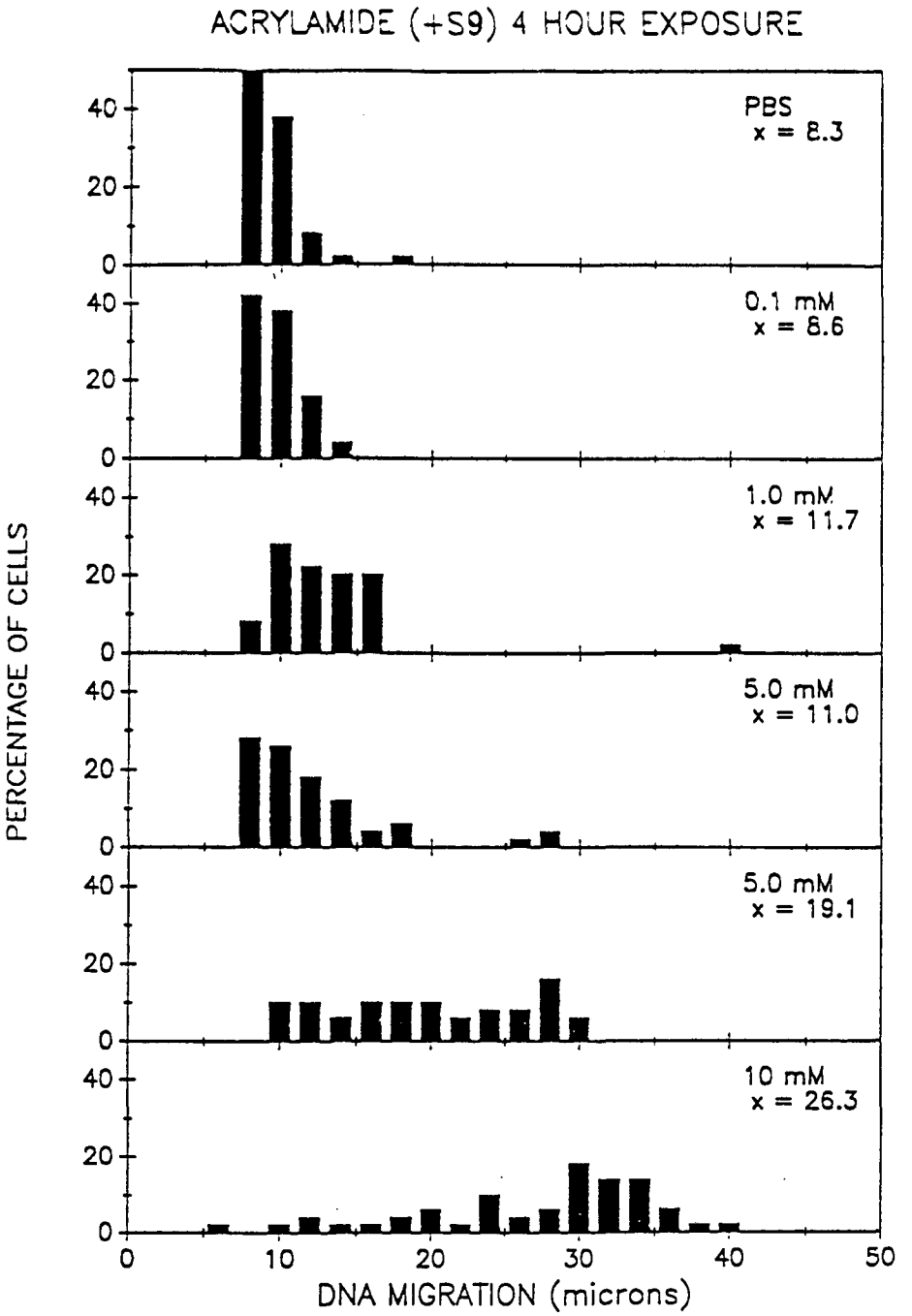

Figure 16. Dose Dependent Distribution of DNA Migration Lengths for CHO Cells Exposed to Acrylamide for 4 Hours in the Presence of Rat Liver S9 Mix. Data are for 50 cells per sample. The width of each bar represents 2 micron intervals. X = group mean DNA migration.

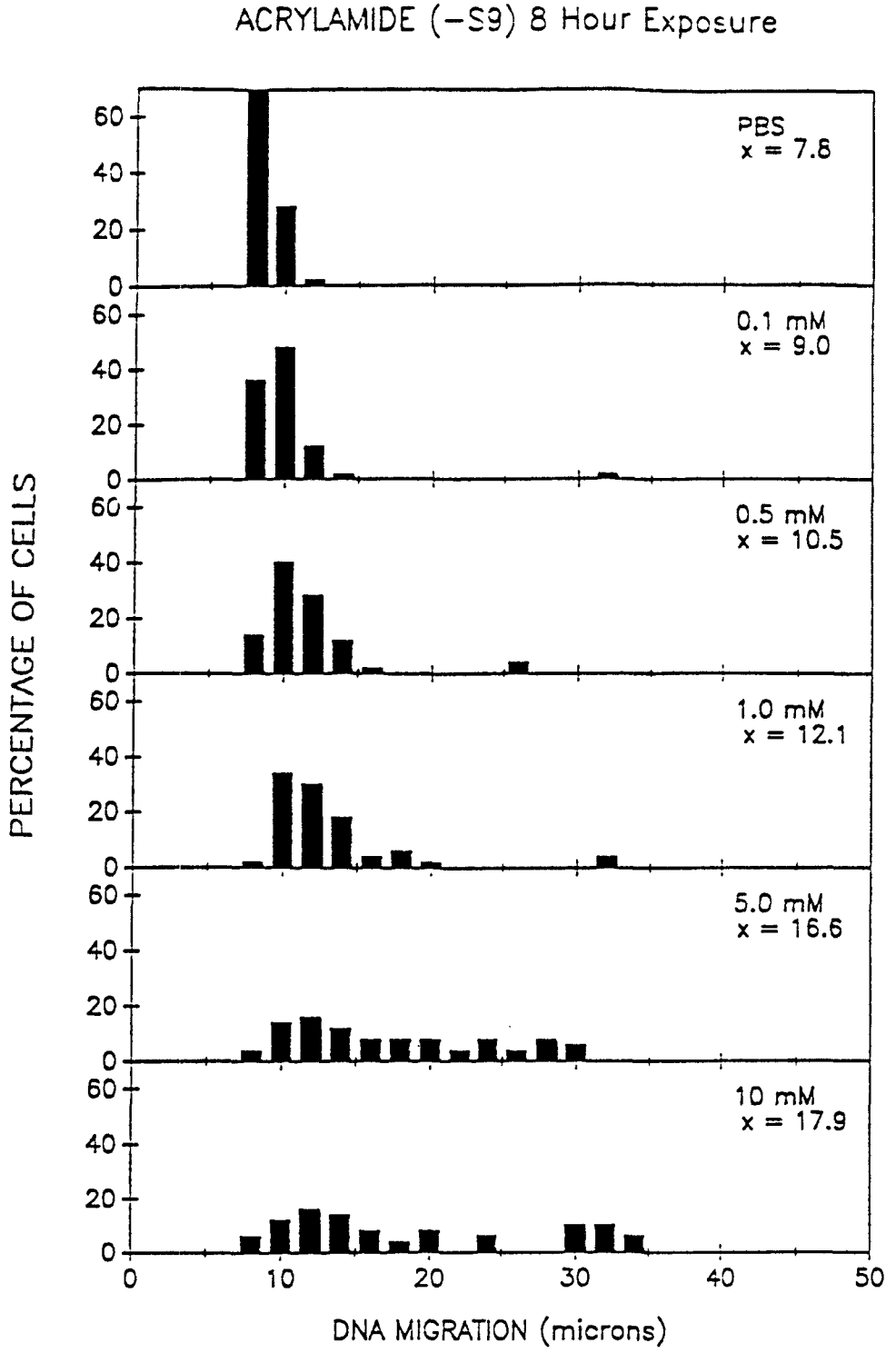

Figure 17. Dose Dependent Distribution of DNA Migration Lengths for CHO Cells Exposed to Acrylamide for 8 hours in the Absence of Rat Liver S9 Mix. Data are for 50 cells per sample. The width of each bar represents 2 micron intervals. X = group mean DNA migration.

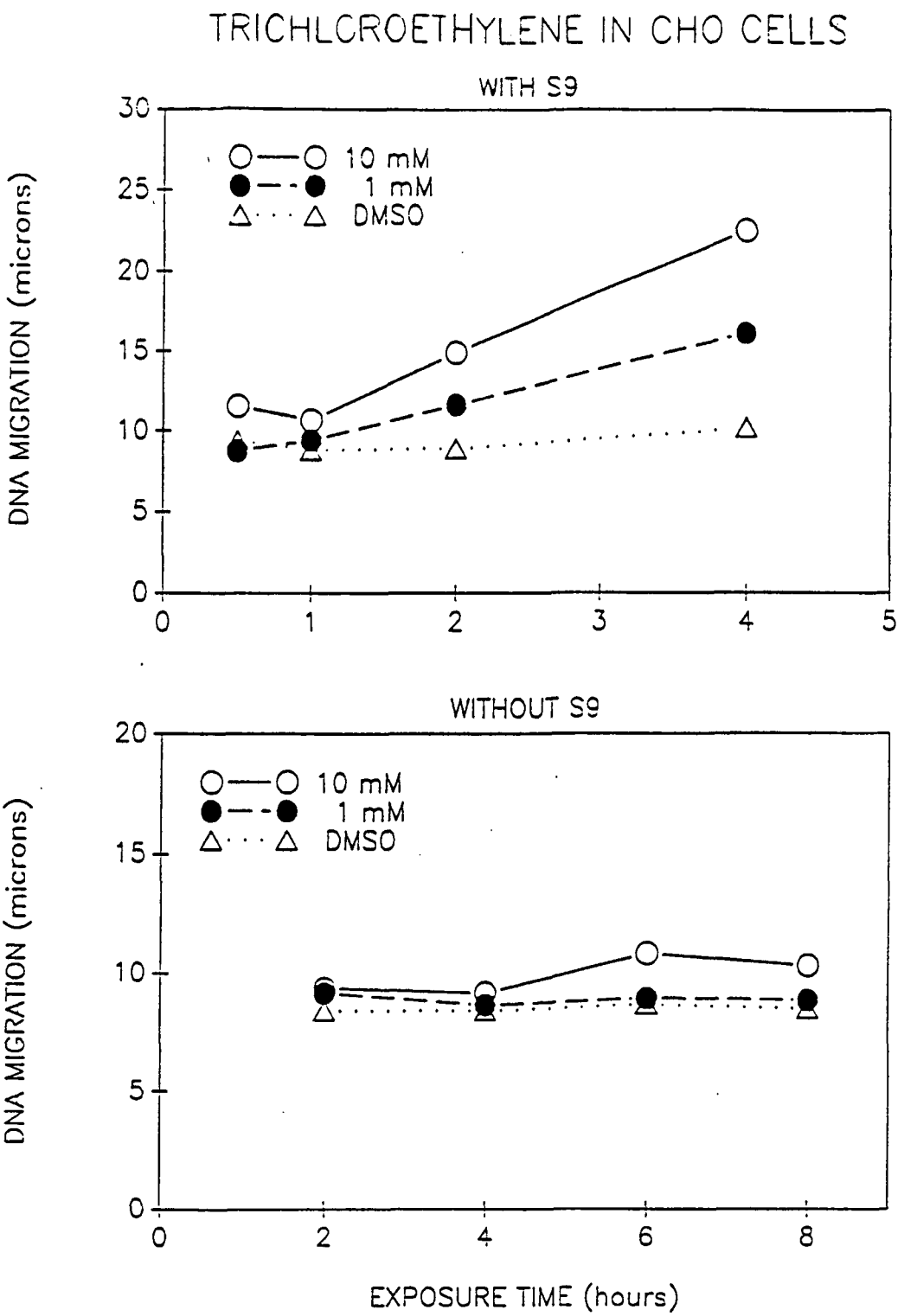

Figure 18. Mean DNA Migration for CHO Cells Exposed to Trichloroethylene as a function of Sample Time in the Presence or Absence of Rat Liver S9 Mix. Each point represents the mean and standard error of the mean for duplicative cultures. For each culture 25 cells were scored.

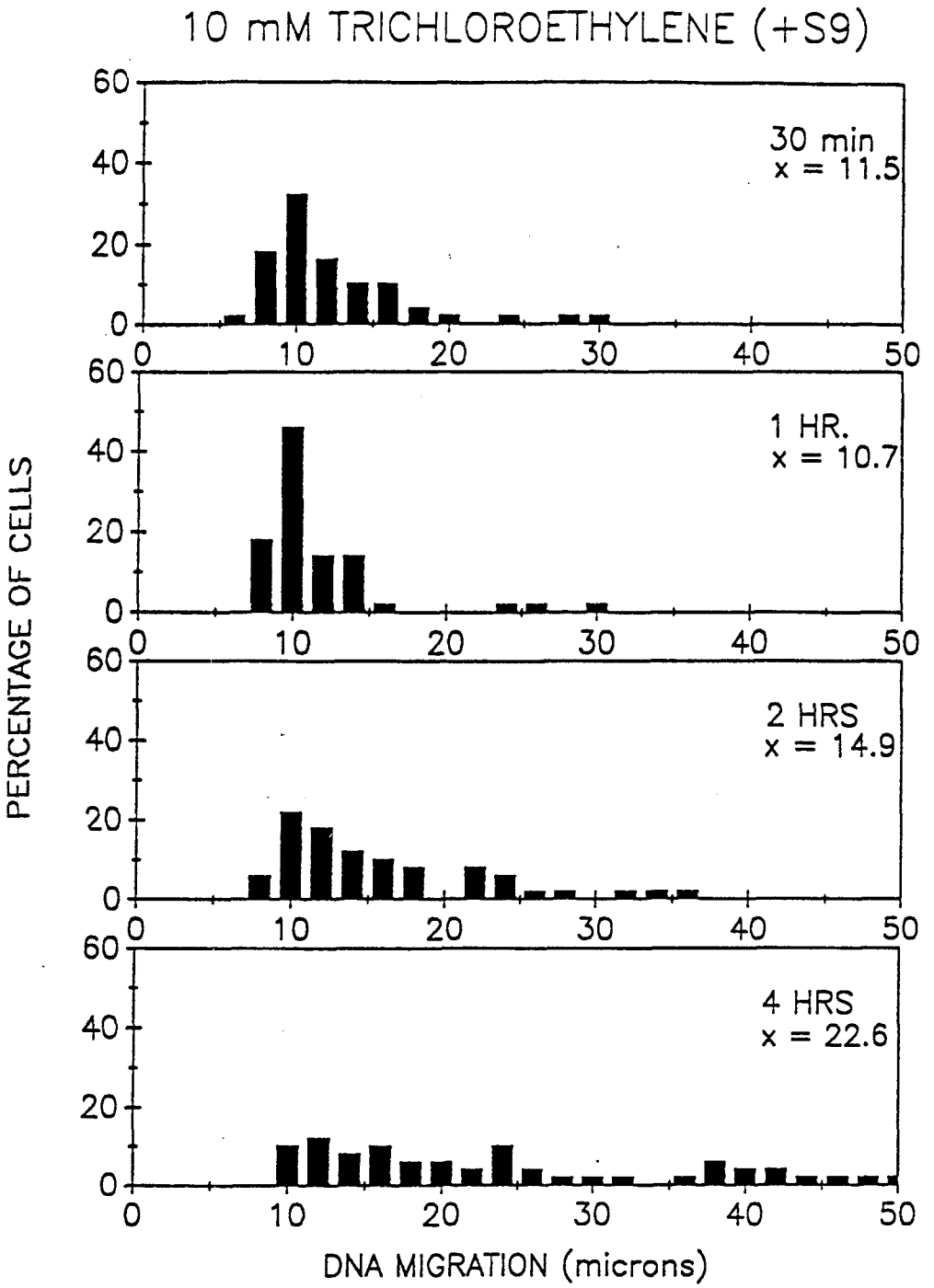

Figure 19. Sample Time Dependent Distribution of DNA Migration Patterns for CHO Cells Exposed to 10mM Trichloroethylene in the Presence of Rat Liver S9 Mix. Data are for 50 cells per sample. The width of each bar represents 2 micron intervals. X = group mean DNA migration.

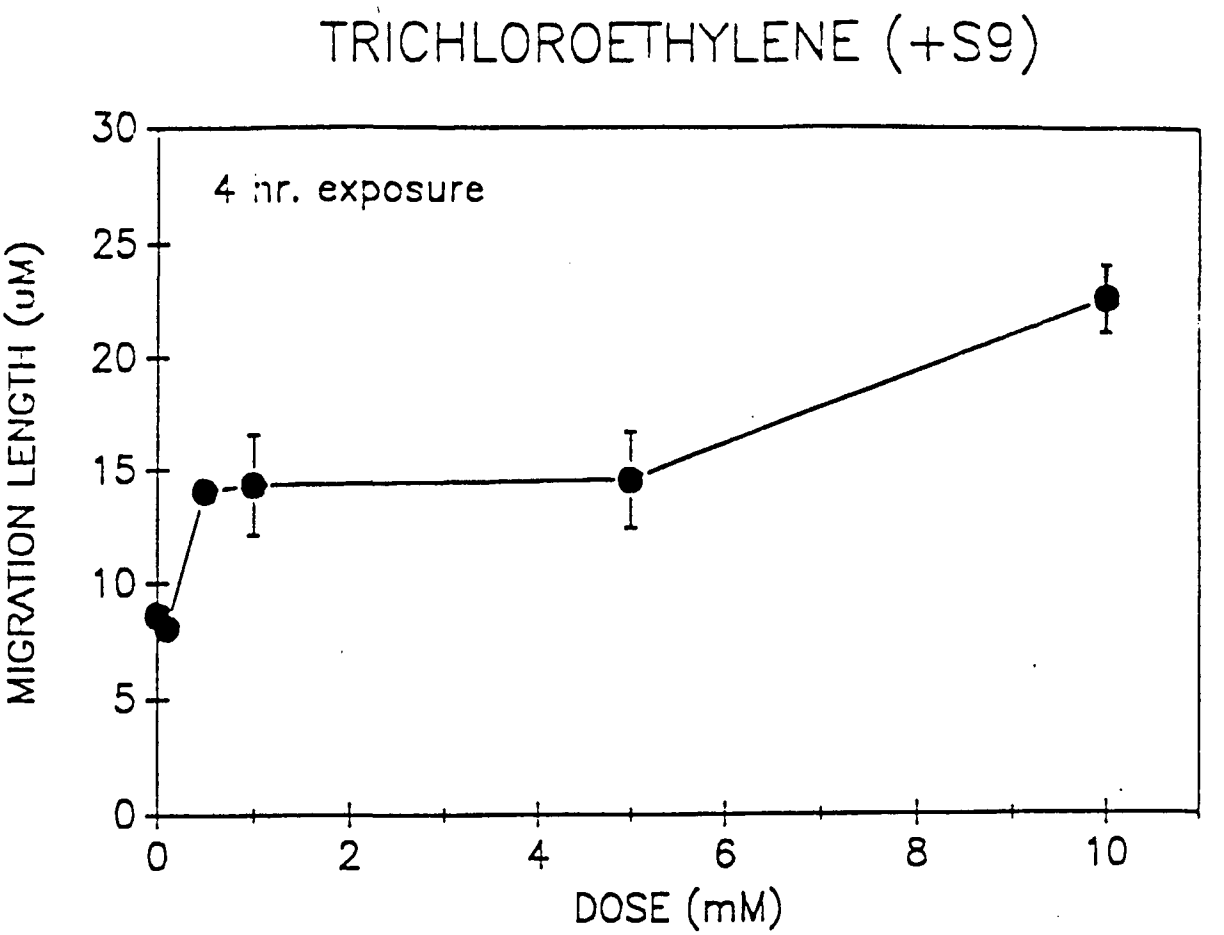

Figure 20. Mean DNA Migration for CHO Cells Exposed to Trichloroethylene as a Function of Dose in the Presence (4 Hour Treatment) of Rat Liver S9 Mix. Each point represents the mean and standard error of the mean for duplicate cultures. For each culture 25 cells were scored.

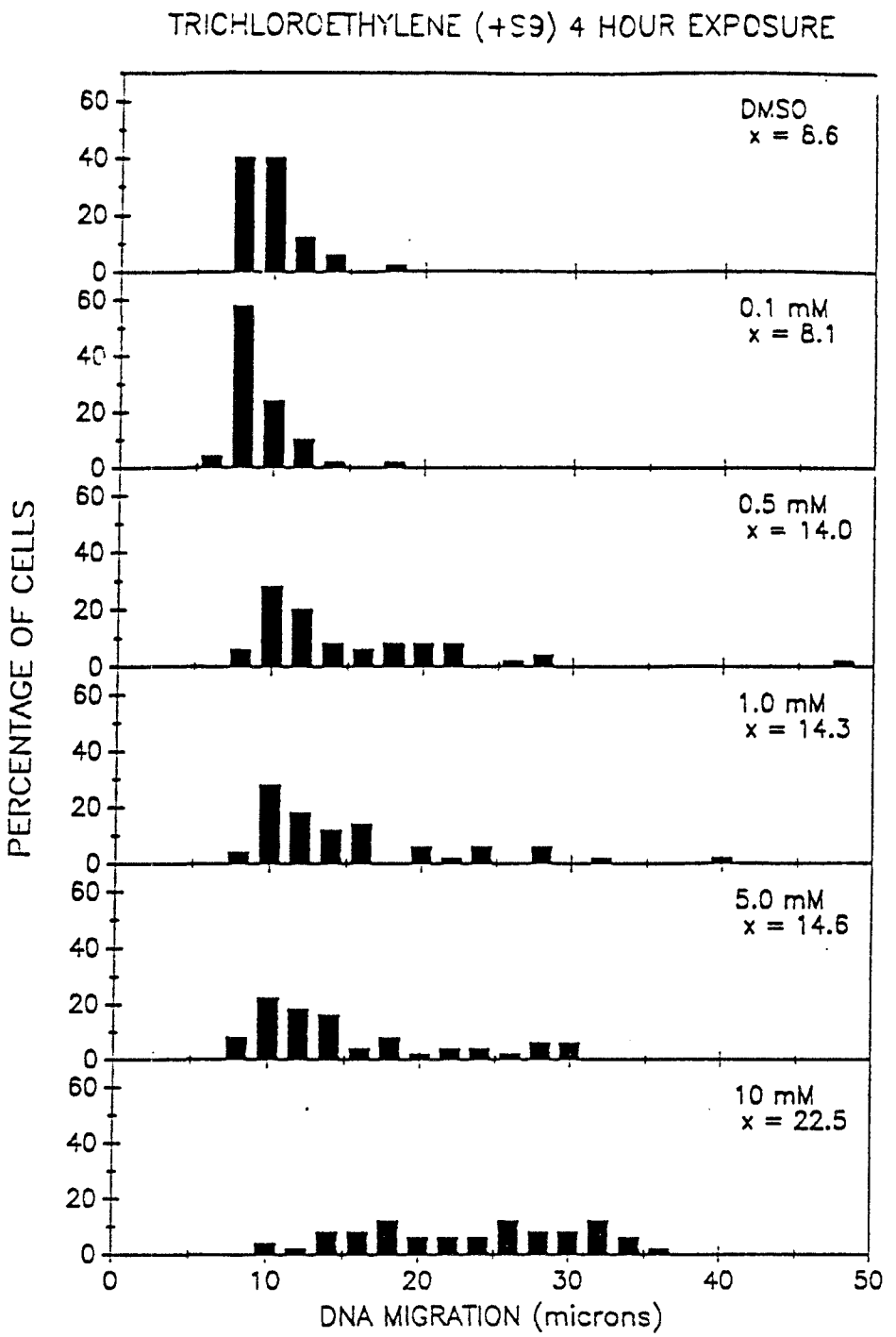

Figure 21. Dose Dependent Distribution of DNA Migration Lengths for CHO Cells Exposed to Trichloroethylene for 8 Hours in the Presence of Rat Liver S9 Mix. Data are for 50 cells per sample. The width of each bar represents 2 micron intervals. X = group mean DNA migration.

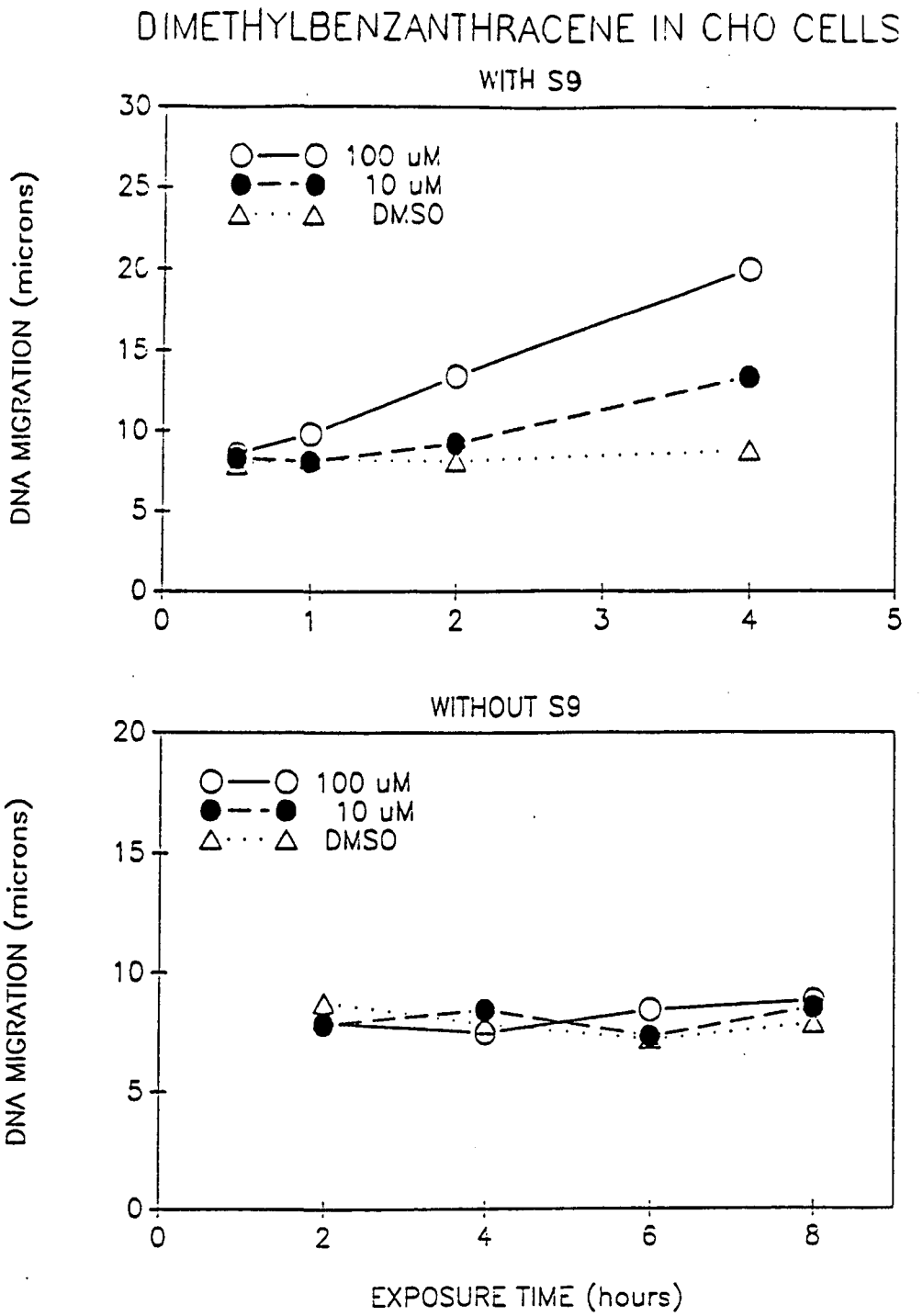

Figure 22. Mean DNA Migration for CHO Cells Exposed to Dimethylbenzanthracene as a Function of Sample Time in the Presence or Absence of Rat Liver S9 Mix. Each point represents the mean and standard error of the mean for duplicate cultures. For each culture 25 cells were scored.

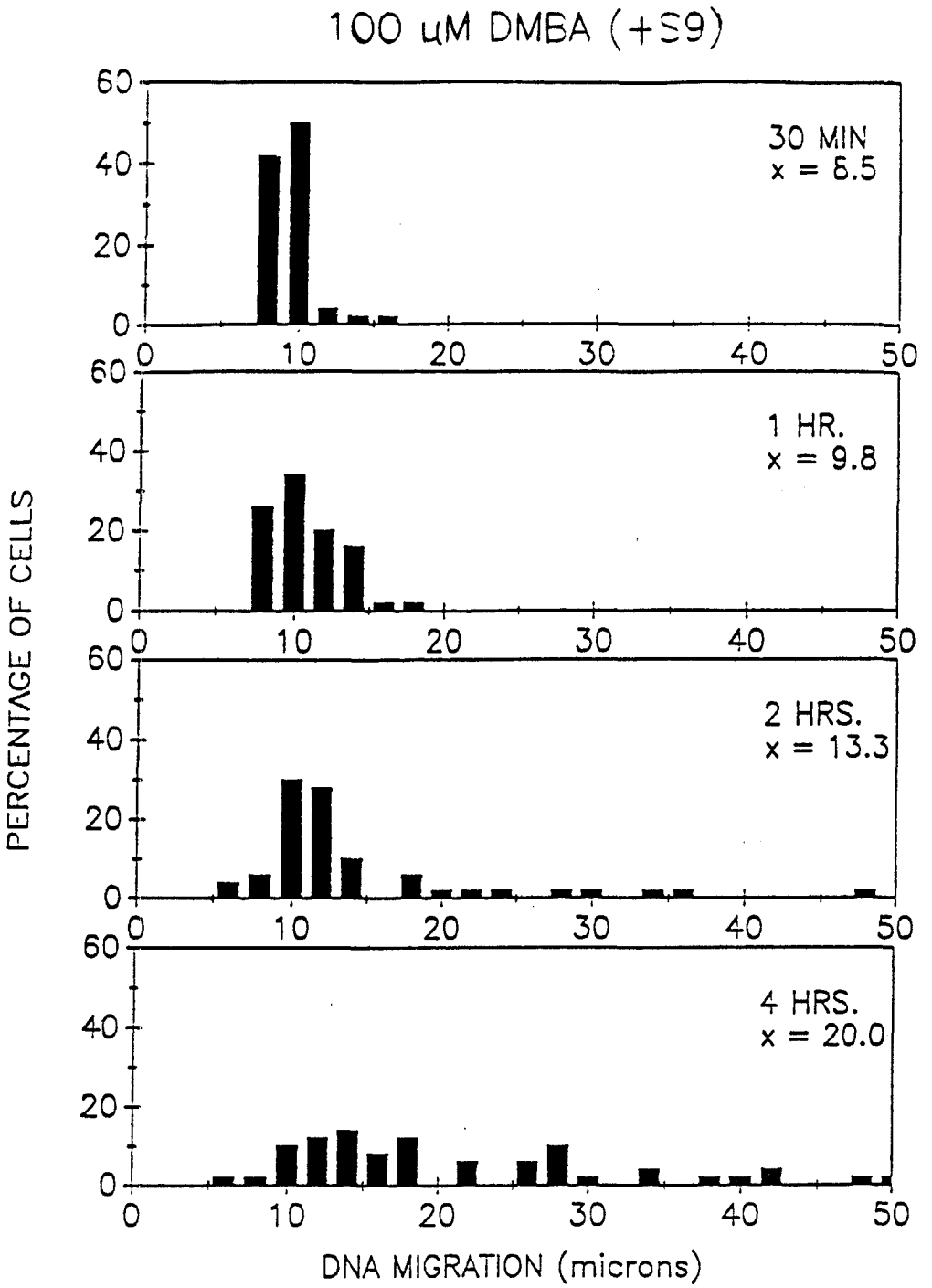

Figure 23. Sample Time Dependent Distribution of DNA Migration Patterns for CHO Cells Exposed to 100 uM Dimethylbenzanthracene in the Presence of Rat Liver S9 Mix. Data are for 50 cells per sample. The width of each bar represents 2 micron intervals. X = group mean DNA migration.

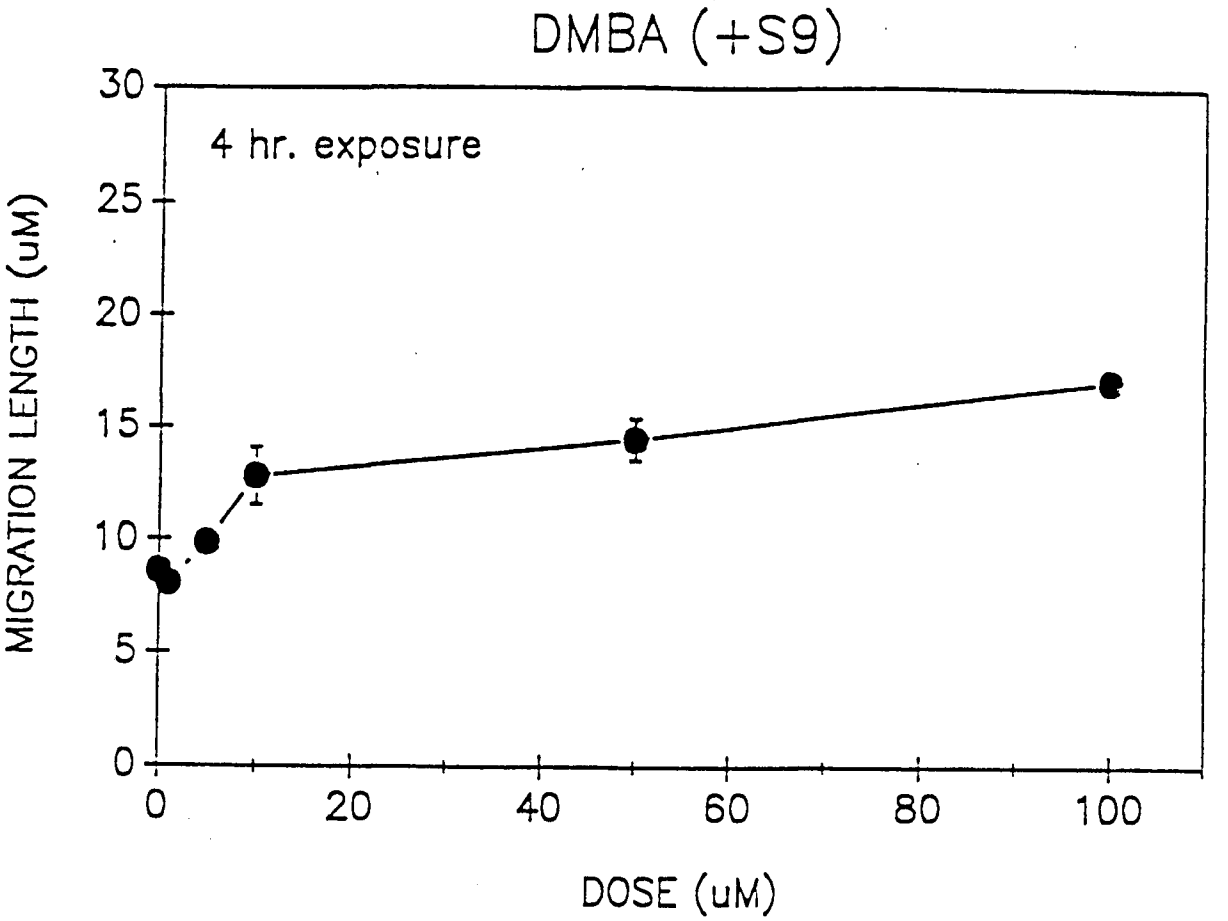

Figure 24. Mean DNA Migration for CHO Cells Exposed to Dimethylbenzanthracene as a Function of Dose in the Presence (4 Hour Treatment) of Rat Liver S9 Mix. Each point represents the mean and standard error of the mean for duplicate cultures. For each culture 25 cells were scored.

in cells exposed to DMBA beginning at the 10 mM dose. The migration histograms for the six doses are shown in Figure 25.

In contrast to the more homogeneous DNA migration patterns observed for cells exposed to ACR, exposure to TCE and DMBA resulted in a more heterogenous intercellular response, with the majority of the cells widely distributed in terms of migration length. Several explanations are possible for this heterogeneity in DNA damage: individual cells may vary in their permeability to the S9-dependent active metabolite(s) of TCE and DMBA, in the accessibility of DNA to the damaging species and/or in repair capacity or in other mechanisms which either enhance or diminish the effects of the compound. These results are consistent with ACR and a metabolite ACR having genotoxic activity and with TCE and DMBA requiring metabolic activation to reactive forms.

5.2.4 In Vitro rodent Hepatocyte Assay: Currently, one of the primary in vitro methods for ascertaining the ability of chemicals to induce DNA damage involves an evaluation of DNA repair synthesis (so-called unscheduled DNA synthesis or UDS) in rodent hepatocytes (Butterworth et al., 1987). This technique is based on an autoradiographic determination of the incorporation of tritiated thymidine into DNA repair sites. Rodent hepatocytes are used because the target cells themselves are metabolically competent, eliminating the need for an exogenous source of metabolic activity. The primary rat hepatocyte UDS assay is considered to be an excellent method for detecting the genotoxic activity and potential carcinogenicity of chemicals (Probst et al., 1980; Williams et al., 1982; Mitchell et al., 1983). However, the very nature of the technique limits its detection generally to those lesions requiring long patch repair, involves the use of radioactive precursors to DNA and requires an extended processing time. Furthermore, based on statistical grounds, the assay is reported as insensitive to weak mutagens (Margolin and Risko, 1988). Based on these considerations, we have begun studies to evaluate the applicability of the SCG assay to a detection of DNA damaging agents using the in vitro rodent hepatocyte cell system. The data presented here comes from an initial experiment involving cyclophosphamide (CP), a well-known alkylating agent requiring metabolic activation.

Mouse hepatocytes were freshly isolated for each test by a two step in situ perfusion of the liver of B6C3F1 male mice (10 - 12 weeks old age, Taconic Farms, Germantown, NY). The procedure used has been previously described for obtaining hepatocytes from rats (Williams, 1977; Butterworth et al., 1987), as modified for the smaller animals (McQueen et al., 1981; Maslansky and Williams, 1982). The liver was initially perfused with 0.5 mM EGTA in Ca_2+, Mg_2+ free Hanks balanced salt solution for 3 minutes at 6 ml/min. This was followed by collagenase (type IV,

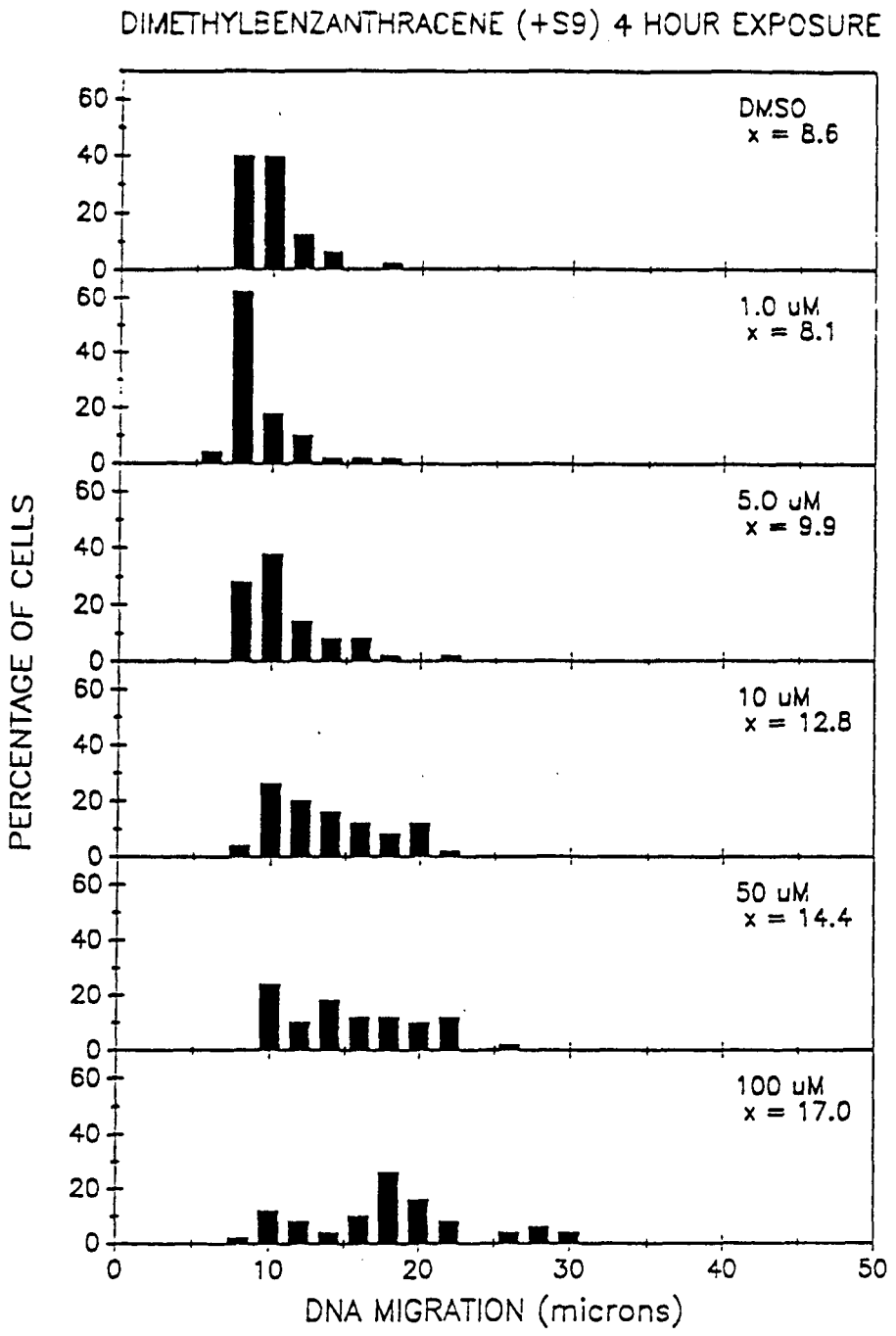

Figure 25. Dose Dependent Distribution of DNA Migration Lengths for CHO Cells Exposed to Dimethylbenzanthracene for 8 Hours in the Presence of Rat Liver S9 Mix. Data are for 50 cells per sample. The width of each bar represents 2 micron intervals. X = group mean DNA migration.

Sigma, 540 U/mg, Lot No. 78F-6814: 100 U/ml of Williams Medium E) for 5 -7 minutes at 5 ml/min. The viability of the cells was determined by trypan blue dye exclusion and only preparations with a cell viability greater than 80% were used. Approximately 1.5 X 10^5 viable cells were plated in a tissue culture plate (6 wells/plate, 3.5 X 1.0 cm, Limbro; Flow Laboratories). The cells were allowed to attach for 1.5 - 2 hr in 2 ml Williams Medium E (WME, GIBCO) supplemented with 10% fetal calf serum (GIBCO) and 2 mM glutamine (GIBCO) at 37°C in a 95% air/5% CO_2 incubator. At the end of this attachment interval, the cultures were washed once with WME.

In the time course study, cultures were exposed to 25 or 250 ug/ml CP for 1 to 8 hr. The selection of these doses was based on published UDS results and on initial toxicity studies indicating that hepatocyte viability was not decreased below 80%. For a dose response evaluation, cultures were exposed to 0.8, 2.5, 8.0, and 25 ug/ml CP for 6 hours. Control and treated cells were washed once with PBS (pH 7.4) and harvested by gentle scrapping with a polyethylene scrapper into cold PBS containing 4 mM EDTA. Viability of the cell suspension was determined by the Trypan blue exclusion test and doses which resulted in more than a 20% decrease in viability were not evaluated. The suspensions were kept on ice until mixed with low melting agarose for the SCG technique.

Identification of Parenchymal versus Nonparenchymal Cells: In pilot experiments to evaluate the applicability of the SCG technique to the in vitro hepatocyte cell culture system, cells with a wide range of diameters were noted. A determination of cell diameters (i.e., the diameter of the nuclear mass), under conditions where DNA unwinding but not DNA migration was permitted, indicated that cells with two discrete size distributions were present. In general, after the unwinding period, approximately 94% of the cells had a nuclear diameter of 6 to 10 um, while the remaining 10% had a diameter of 3 to 5 um. About a third of the larger sized nuclei appeared as doublets, indicating that they had originated from binucleate cells. Based on their size and morphology, nuclei with a diameter above 6 um were classified as parenchymal cells while nuclei smaller than 5 um were classified as nonparenchymal cells.

Time Course Study: Beginning at the two hour sample time, the incubation of mouse hepatocytes with CP at 25 and 250 ug/ml resulted in a significant increase in the length of DNA migration in parenchymal cells (Figure 26). After 4 hours of exposure, the level of DNA damage was not significantly different between the two doses and appeared to have saturated. An analysis of the distribution of migration patterns among individual parenchymal cells indicated an increasingly homogeneous response with increasing sample time (Figure 27). Viability in this experiment remained above 90%.

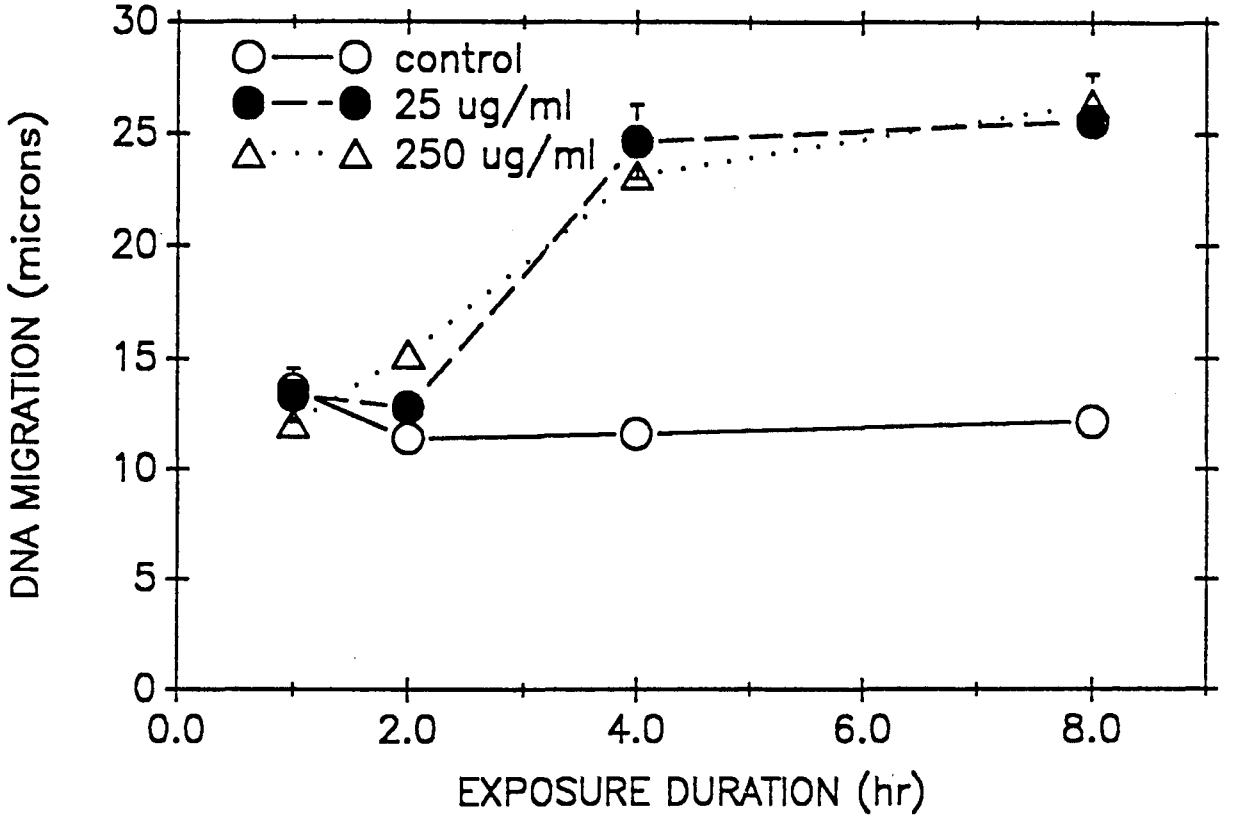

Figure 26. Time Course for the Induction of DNA Damage in Primary Mouse Parenchymal Cells by Cyclophosphamide. Data are presented as the mean and standard error of the mean for triplicate cultures. 25 cells were scored in each culture.

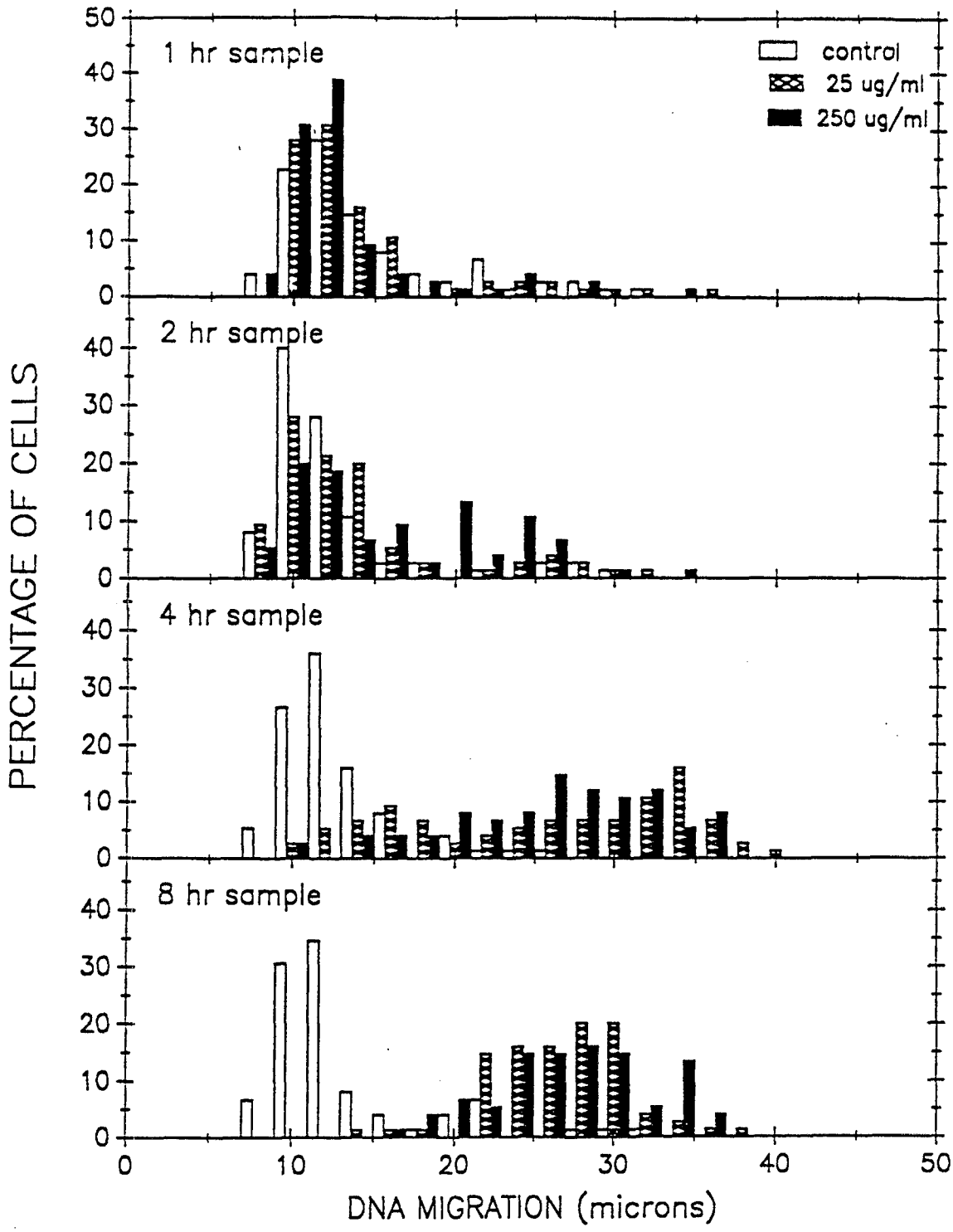

Figure 27. Distribution of DNA Migration Lengths among Individual Primary Mouse Parenchymal Cells Exposed to Cyclophosphamide as a Function of Sample time. Data for each sample based on 75 cells. The width of each bar represents 2 microns.

Dose Response Study: Based on this initial kinetic study, a dose-response experiment was conducted using a 6 hour exposure period and CP at doses between 0.8 and 25 ug/ml. At the highest dose tested, viability was not decreased below 93%. Under these conditions, CP induced a progressive increase in DNA migration length, indicating a dose-dependent generation of DNA strand breaks (Figure 28). An analysis of the distribution of migration patterns among individual cells indicated that the intercellular distribution of DNA migration patterns was more homogenous with increasing CP dose (Figure 29).

Subsequent to these initial in vitro mouse hepatocyte studies, several additional chemicals, including 2-acetylaminofluorene, 4-acetylaminfluorene, benz(a)pyrene and mitomycin C, have been examined for their ability to induce DNA damage in mouse and/or rat liver cells. Data on 2-acetylaminofluorene and 4 acetylaminofluorene for both mouse and rat hepatocytes are presented in Figure 30.

One of the more interesting aspects of this research was the observation that parenchymal cells, the hepatocytes responsible for the metabolic activation of procarcinogens to carcinogens, could be readily distinguished on the basis of size from nonparenchymal support cells. This suggested that, by analyzing the induction of DNA damage in both cell types concurrently, direct-acting genotoxic chemicals (i.e., those capable of inducing damage without metabolic activation) could be discriminated from chemicals requiring metabolic activation. For procarcinogens, it would be anticipated that DNA damage would preferentially occur in parenchymal cells, with DNA damage in nonparenchymal cells occurring as a result of the release of reactive intermediates into the culture media. For direct-acting chemicals, the level of damage in both cell types should be approximately equal, assuming equal transport of the chemical into the cell and equal repair capacity. Several experiments were conducted to verify this hypothesis. For example, the induction of DNA damage by dimethylnitrosamine (DMN) was highly cell-type specific. A significant increase in DNA migration was detected in parenchymal cells at lower doses and a earlier times than in nonparenchymal cells (Figure 31, 32). Since this chemical is not genotoxic unless metabolically activated, the data support the initial hypothesis. An experiment involving the treatment of rat hepatocytes with ethylmethanesulphonate (EMS), a direct-acting chemical, demonstrates equal levels of DNA migration among the two cell types (Figure 33, 34).

While not exhaustive, these experiments strongly suggest the potential wide-spread applicability of the SCG technique to the in vitro rodent hepatocyte culture system. Not only due the data demonstrate the sensitivity and reproducibility of the assay, but indicate also the ability to distinguish between direct-acting and metabolically-requiring genotoxic agents.

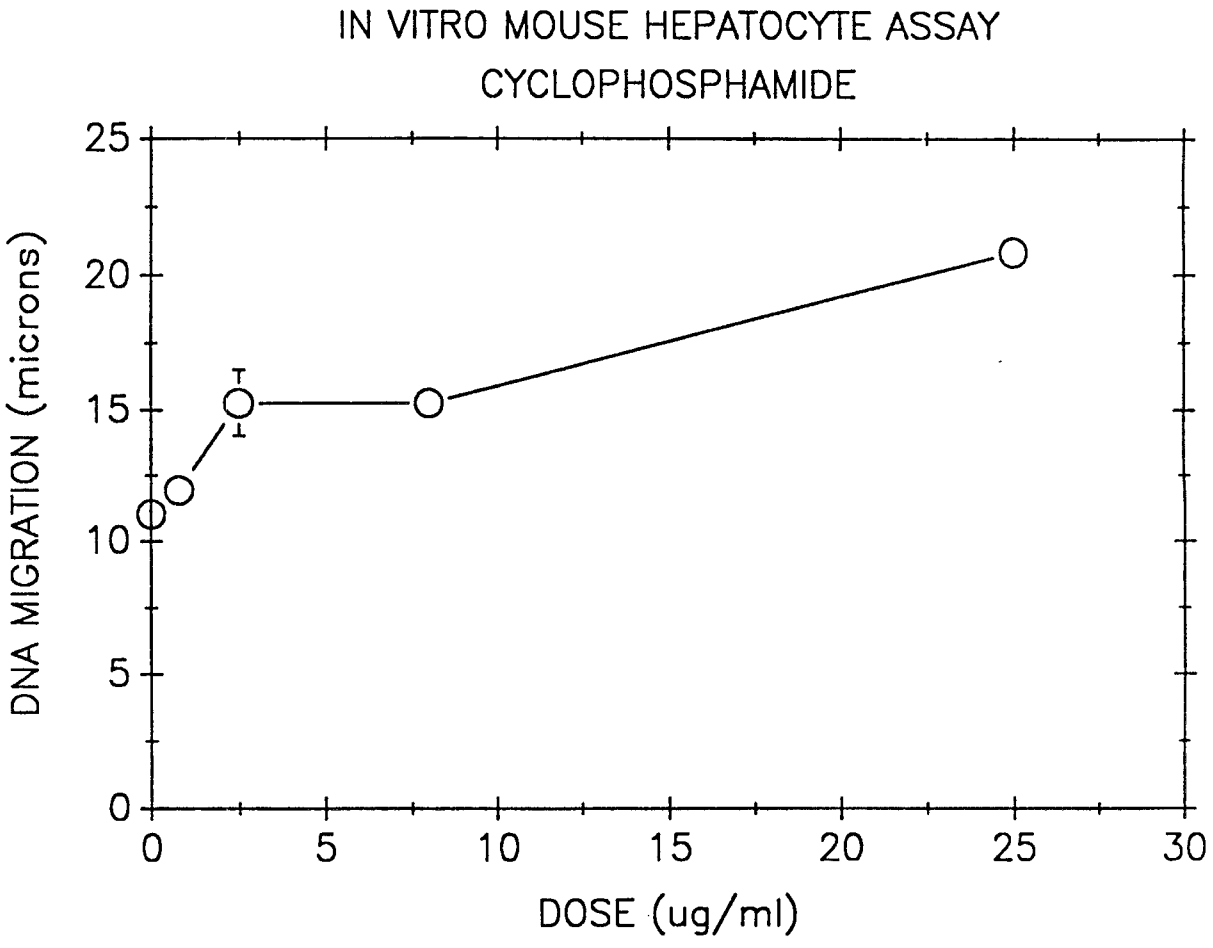

Figure 28. DNA Migration in Parenchymal Cells as a Function of Cyclophosphamide Dose, Sampled after 6 Hours of Treatment. Data are presented as the mean and standard error of the mean for triplicate cultures. 25 cells were scored in each culture.

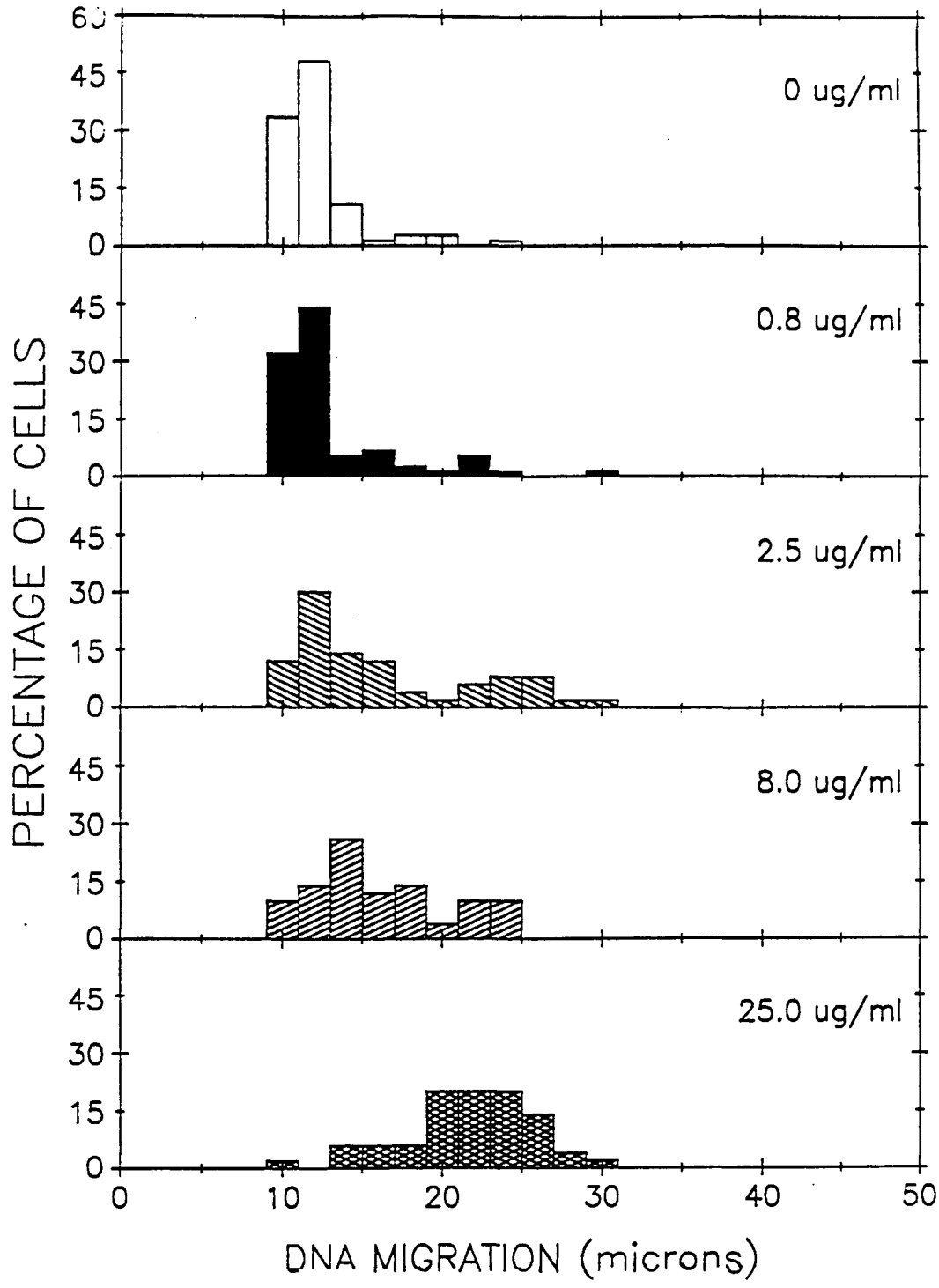

Figure 29. Distribution of DNA Migration Lengths among Individual Primary Mouse Parenchymal Cells Exposed to Cyclophosphamide for 6 Hours. Data for each sample based on 75 cells. The width of each bar represents 2 microns.

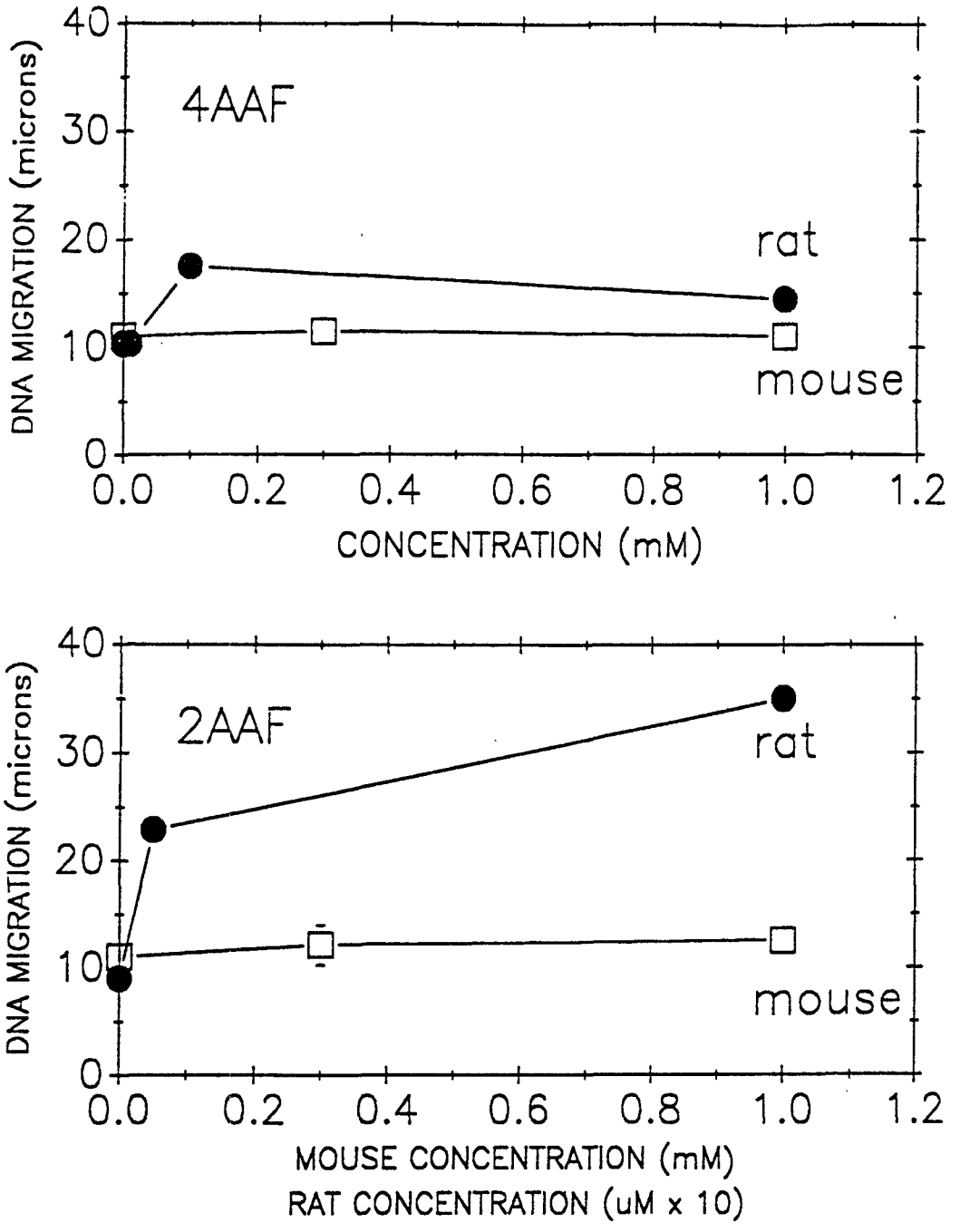

Figure 30. Comparative Analysis of the Induction of DNA Damage in Rat and Mouse Primary Parenchymal Cells by 2-Acetylaminofluorene and 4-Acetylaminofluorene. Data are presented as the mean and standard error of the mean for triplicate cultures. 25 cells were scored in each culture.

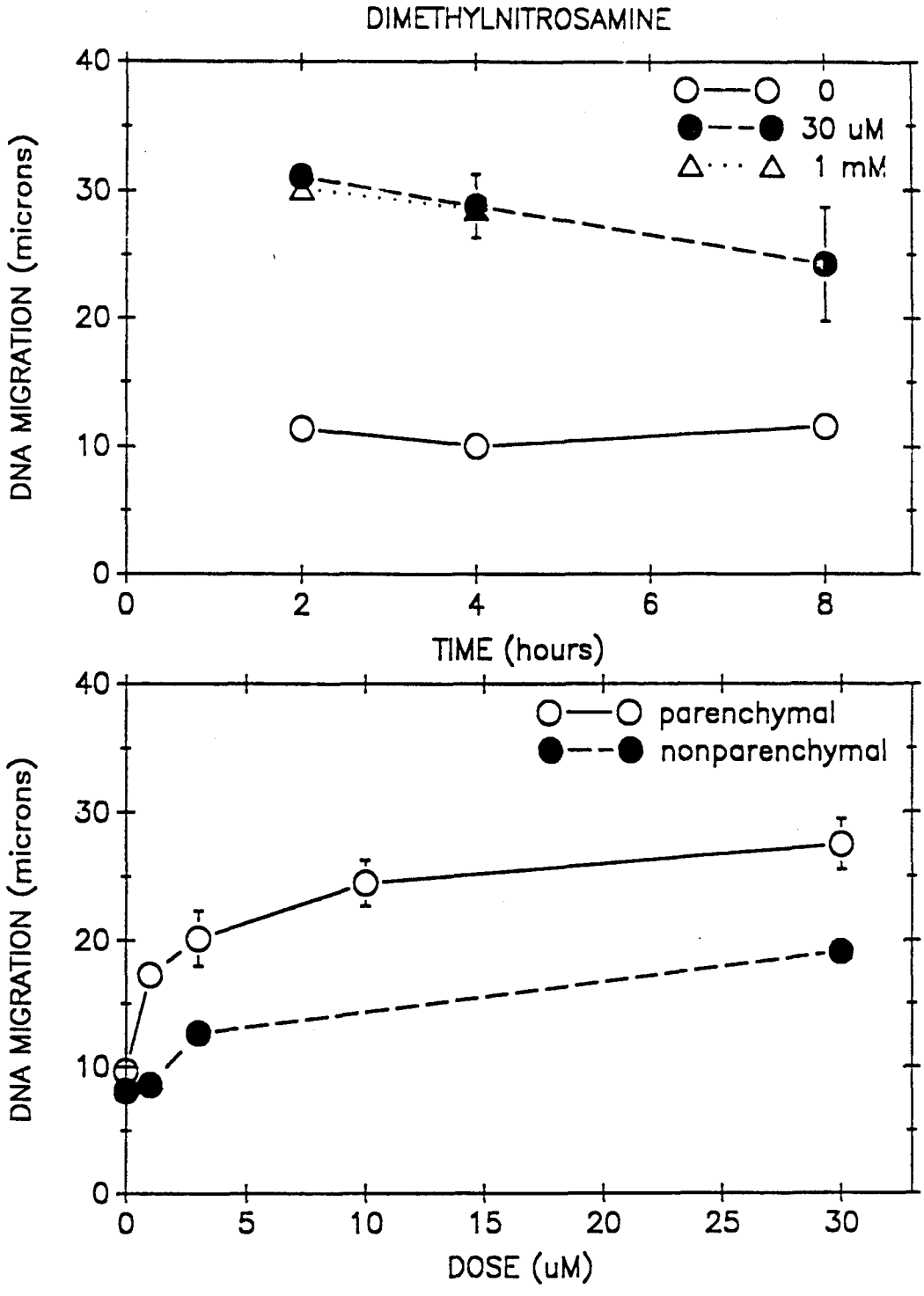

Figure 31. DNA Migration in Mouse Primary Parenchymal and Nonparenchymal Cells Exposed to Dimethylnitrosamine for 6 Hours. Data are presented as the mean and standard error of the mean for triplicate cultures. 25 cells were scored in each culture.

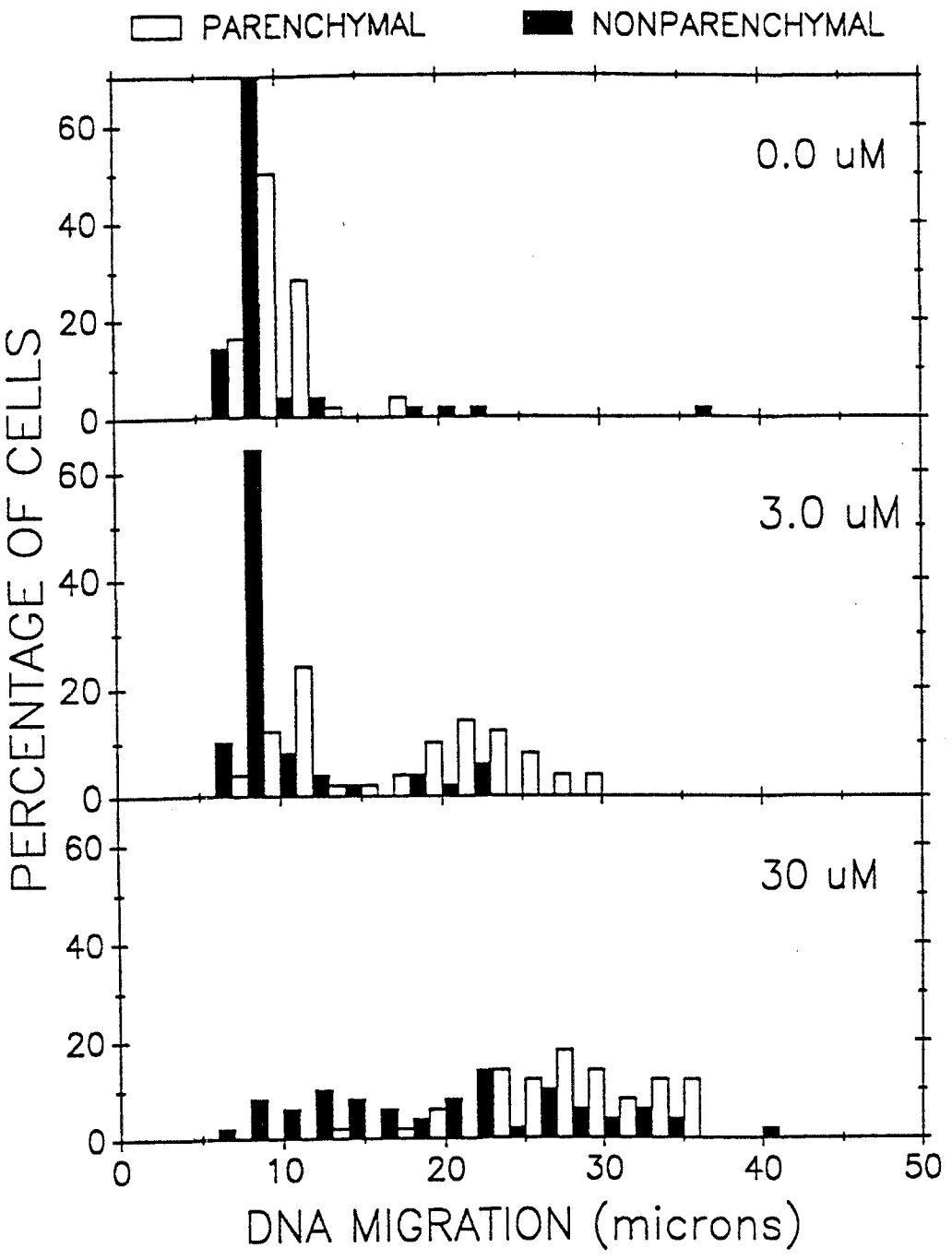

Figure 32. Distribution of DNA Migration Lengths among Individual Mouse Primary Parenchymal Cells Exposed to Dimethylnitrosamine for 6 Hours. Data for each sample based on 75 cells. The width of each bar represents 2 microns.

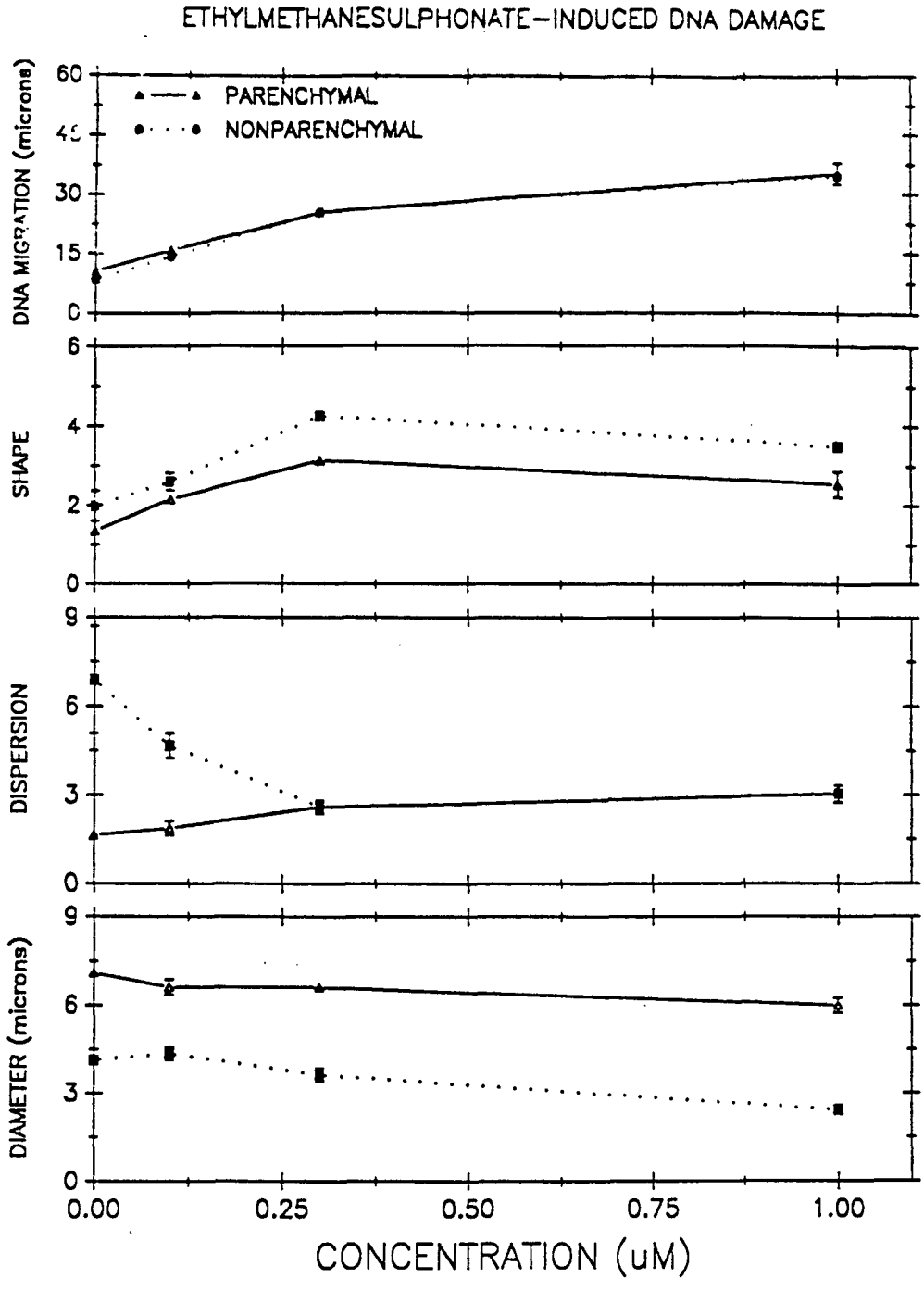

Figure 33. Ethylmethanesulphonate-Induced DNA Damage in Mouse Primary Parenchymal and Nonparenchymal Cells Exposed for 6 Hours. The presence of damage is measured by changes in DNA migration, shape (perimeter squared/area), and dispersion (variance/mean). Data on image diameter are presented to indicate the difference in size between parenchymal and nonparenchymal cells. Data are presented as the mean and standard error of the mean for triplicate cultures. 25 cells were scored in each culture.

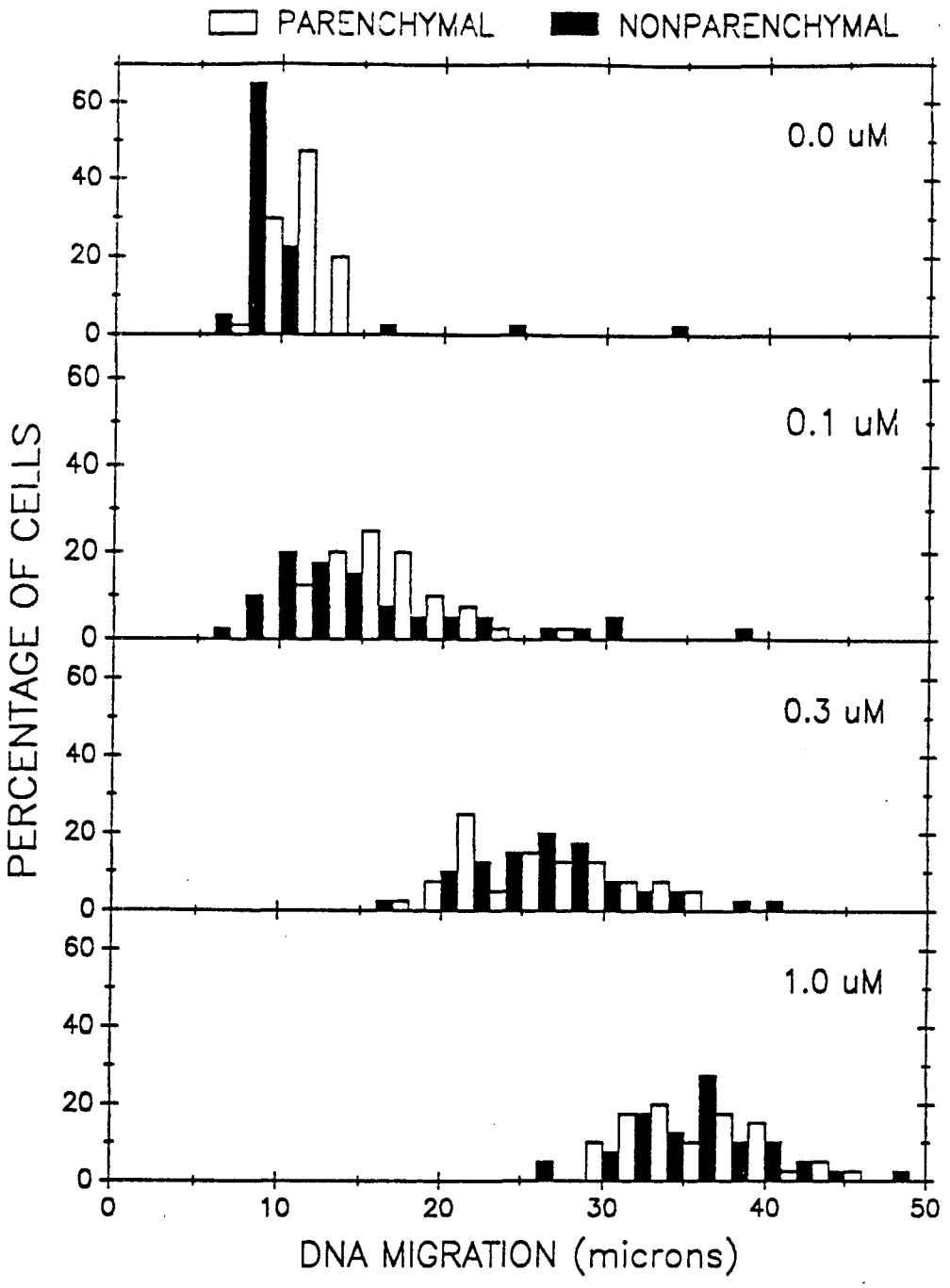

Figure 34. Distribution of DNA Migration Lengths among Individual Mouse Primary Parenchymal Cells Exposed to Ethylmethanesulphonate for 6 Hours. Data for each sample based on 75 cells. The width of each bar represents 2 microns.

6.0 IN VIVO STUDIES

Initially, a series of experiments were conducted to evaluate the ability of ACR, DMBA and trichloroethylene (TCE) to induce DNA damage in mice in four different tissues (e.g., brain, liver, spleen and blood). In these experiments, male B6C3F1 mice were exposed acutely by gavage to 100 mg/kg ACR in PBS, 100 mg/kg DMBA in corn oil or to 1000 mg/kg TCE in corn oil. Groups of mice (4 per group) were killed by etherization at 4 and 24 hours after treatment and peripheral blood, liver, spleen and brain samples obtained from each mouse. These samples were processed with (brain, liver) or without (blood, spleen) collagenase treatment and evaluated for DNA damage using the SCG technique. The doses and the sample times used in this study are based on previous experience with these chemicals in this and other laboratories. Mortality among the treated animals was not anticipated under these experimental conditions and did not occur. Tissue selection was based on technical ease of processing (blood, spleen), on metabolic (liver) and neurological (brain) importance and on the expectations that DNA damage in these tissue would accumulate under multiple exposure conditions. Because of fairly extensive tissue and experiment to experiment differences in control migration patterns, the percentage of increase is discussed rather than the actual raw data.

Four hours after treatment with ACR, cells from all four organs/tissues exhibited a significant increase in DNA migration, with liver cells exhibiting the greatest percentage increase in response. By 24 hours after treatment, only blood leukocytes (PBL) still appeared to exhibit an increased level of damage. None of the cells obtained from organs/tissues sampled four hours after treatment with DMBA (100 mg/kg) exhibited an increase in DNA migration. However, at 24 hours after treatment, cells from all but brain exhibited a significant increase in DNA migration, with spleen cells exhibiting the greatest response. Four hours after treatment with TCE (1000 mg/kg), cells from all four organs/ tissues exhibited a significant increase in DNA migration, with spleen cells exhibiting the greatest response. By 24 hours after treatment, all tissues exhibited DNA migration patterns indistinguishable from that observed for control mice.

These pilot studies demonstrated, not surprisingly, that the level of DNA damage induced by these chemicals was agent, organ and sample time dependent. They also demonstrate the utility of the approach and the feasibility of detecting DNA damage in individual cells isolated from different organs of mice. However, the range of variation among cell samples from control mice (approximately 2 to 3 fold) was extremely disappointing and suggested the need to characterize the processing of in vivo tissues in greater depth. Based on conversations with other scientists while at the International Environmental Mutagen Society meeting in Cleveland, the first technical factor

evaluated was the effect of the collagenase treatment used to isolate single cells from the brain and liver. The second factor was the presence of calcium chelators in the mincing and lysing solutions. Finally, the third factor considered was the influence of blood contamination on the resulting DNA migration patterns.

6.1 Collagenase Treatment

In this experiment, brain and liver tissue were removed from a single mouse and minced, either in the presence or absence of collagenase prior to SCG analysis. The resulting data indicated that collagenase treatment resulted in a significant increase in DNA migration (approximately 50% greater) and that mincing alone was sufficient for ensuring an adequate sample of single cells from every tissue tested (including spleen, liver, brain, testis).

6.2 Calcium Chelators

In the original protocol developed by Singh and his colleagues (Singh et al., 1988), calcium chelators such as EDTA or EGTA were omitted from the media solutions because it did not appear as if DNA damage resulting from the presence of nucleases occurred at any time during the processing of human leukocytes. However, liberation of such nucleases is more likely during the mincing and handling of in vivo tissues, resulting in increased levels of DNA damage. In several experiments, this possibility was evaluated by adding various concentration of EDTA or EGTA, two calcium chelators, to the mincing solutions used during the processing of liver tissue. In Figure 35, group mean migration lengths from a representative experiment involving EDTA are presented. These data demonstrate the importance of the addition of a calcium chelator to the mincing solutions.

The adverse impact of blood in the lysing solution and its correction by the addition of DMSO has already been discussed. Based on the results of these experiments, the protocol has been modified by the addition of 20 mM EDTA (or EGTA) to the mincing solutions, by the addition of 10% DMSO to the lysing solution, and by the omission of collagenase from the mincing solution.

6.3 A Kinetic Study of Acrylamide-Induced Organ Specific Levels of Damage

After the several experiments discussed above to improve our in vivo tissue sampling procedures, we decided to return to an evaluation of organ-specific levels of DNA damage induced in vivo in mice by an environmentally important pollutant with genotoxic activity. The chemical we selected to evaluate first was acrylamide (ACR). Studies with TCE were started but not

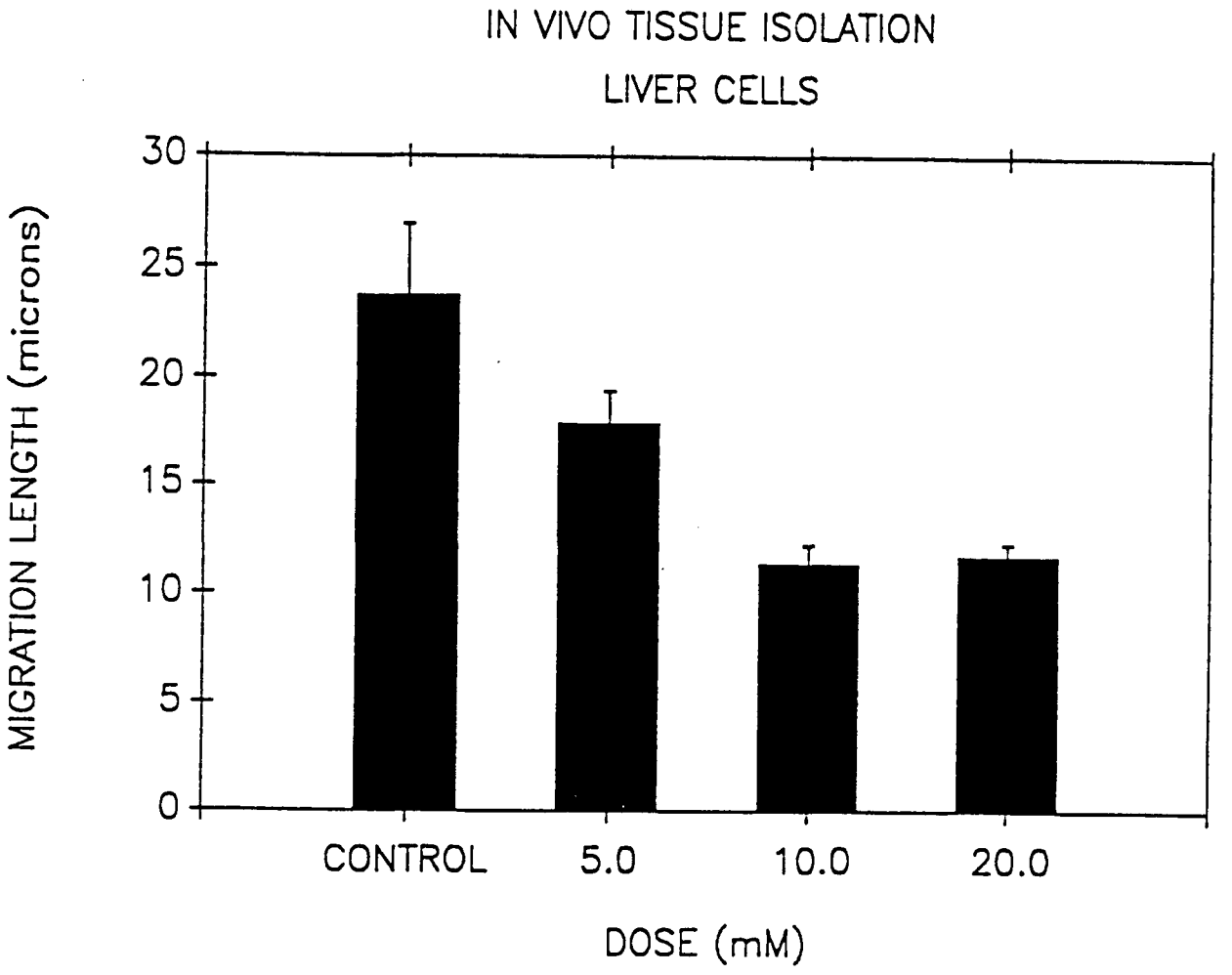

Figure 35. Effect of EDTA on the DNA Migration for Liver Cells Processed from a Control Mouse. Bars indicate group means and the standard error of the mean for 25 cells per sample.

completed due to technical difficulties already discussed (e.g., problems with slide batches, coplin jars, electrophoresis buffer pH, etc.) and a decision to focus additional resources on the collection of human data and on animals inhabiting hazardous waste sites.

Briefly, male B6C3F1 mice (10 - 13 weeks of age, 25 to 32 gm in body weight, 4 mice per group) were gavaged with 10 and 100 mg/kg ACR in PBS. At 3, 6, 12, 24 and 48 hours after treatment, groups of mice were killed by CO_2 asphyxiation, and samples of liver, spleen and testis were removed from each mouse and stored on ice in Hank's buffer containing 20 mM EDTA. Blood samples were collected by adding 5 uL to RPMI-1640. The samples of liver, spleen and testis were minced, while the blood sample was centrifuged to recover the leukocytes. An aliquot of cells were mixed with LMA and placed on microscope slides as described earlier. The cells were electrophoresed for 20 minutes, stained with ethidium bromide and 25 cells per cell type per sample were evaluated for DNA migration using the Quantimet 520 image analyzer. Total leukocytes were scored from blood, splenocytes from spleen, parenchymal and nonparenchymal cells from liver, while the analysis of damage in testis was limited to diploid cells. Cell viability measurements, as described earlier, were also conducted on each tissue and viability was routinely above 90%.

Based on an analysis of mean DNA migration patterns, treatment with ACR induced a significant increase in DNA damage in all organs at all sample times (Figure 36), the magnitude of which was dose (100 << 10 mg/kg), tissue blood leukocytes > spleen = liver > testis) and sample time (maximum response at 12 hours) dependent. In liver, both parenchymal and nonparenchymal cells exhibited a similar increase in DNA damage. The corresponding distributional data are presented, by tissue, in Figures 37-41. This analysis indicates that even at 10 mg/kg, a small but significant subpopulation of cells in each tissue expressed some damage. The analysis also indicates that the extent of DNA migration shifted in almost all cells of the liver, spleen and testis toward control levels by 48 hours. At this delayed sample time, blood leukocytes, however, exhibited greater intercellular heterogeneity in DNA migration patterns. This suggests that the leukocytes in the blood are more variable in repair than cells in the other tissues. These data also demonstrate, under the modified sampling protocol, reproducible control data for each tissue between sample times, and reproducible data among animals at a specific dose of ACR.

7.0 HUMAN STUDIES

Because one of the ultimate goals of this project is to be able to evaluate and compare, where feasible, data obtained on both animal and human populations, several pilot studies were

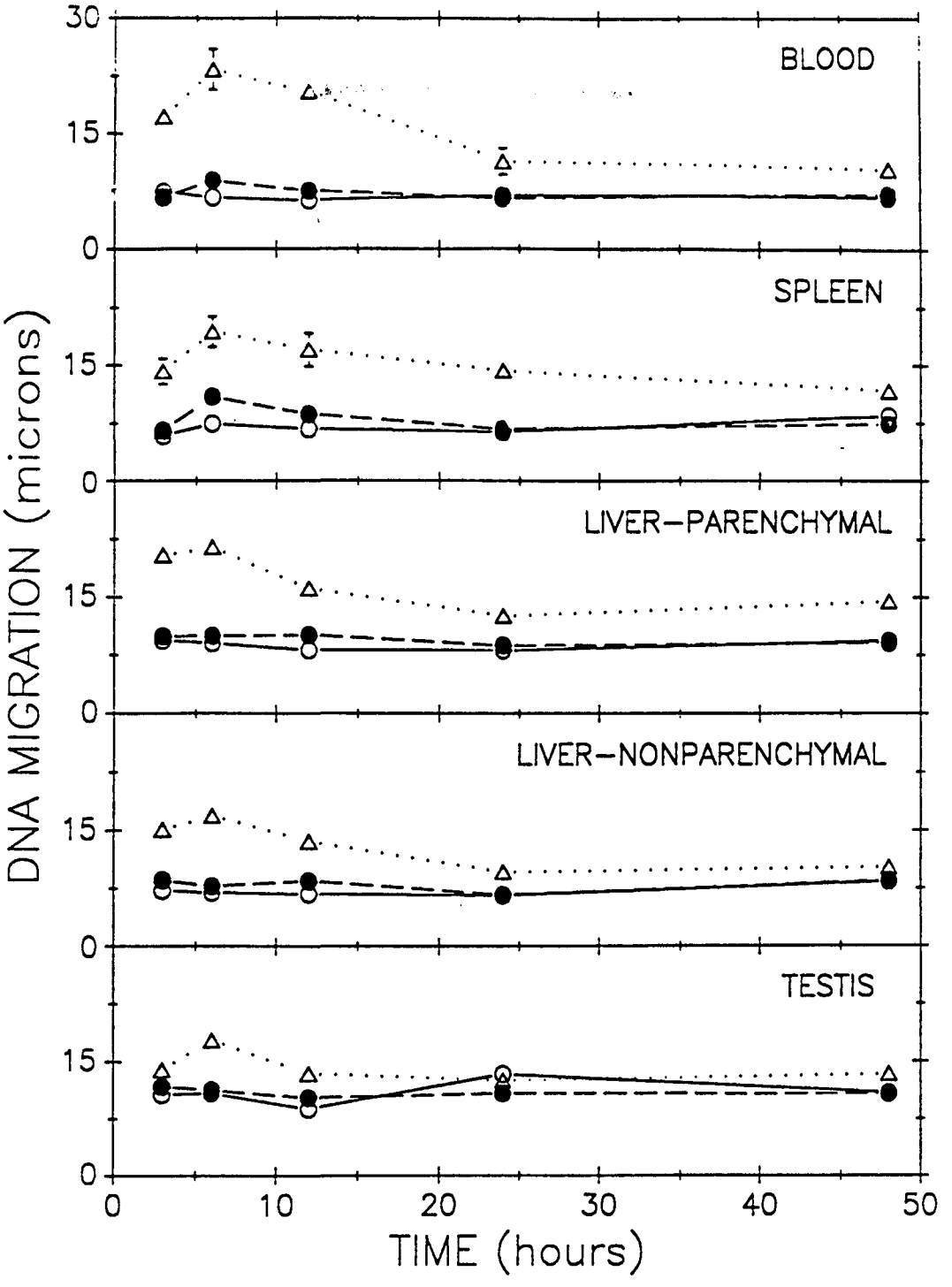

Figure 36. Evaluation of Acrylamide-Induced DNA Damage, as a Function of Sample Time, in Various Tissue of Male B6C3F1 Mice. Data are presented as group mean and the standard error of the mean among 4 animals. 25 cells were scored per tissue. Treatment was by gavage. Open triangles = 100 mg/kg; solid circles = 10 mg/kg; open circles = phosphate buffered saline only.

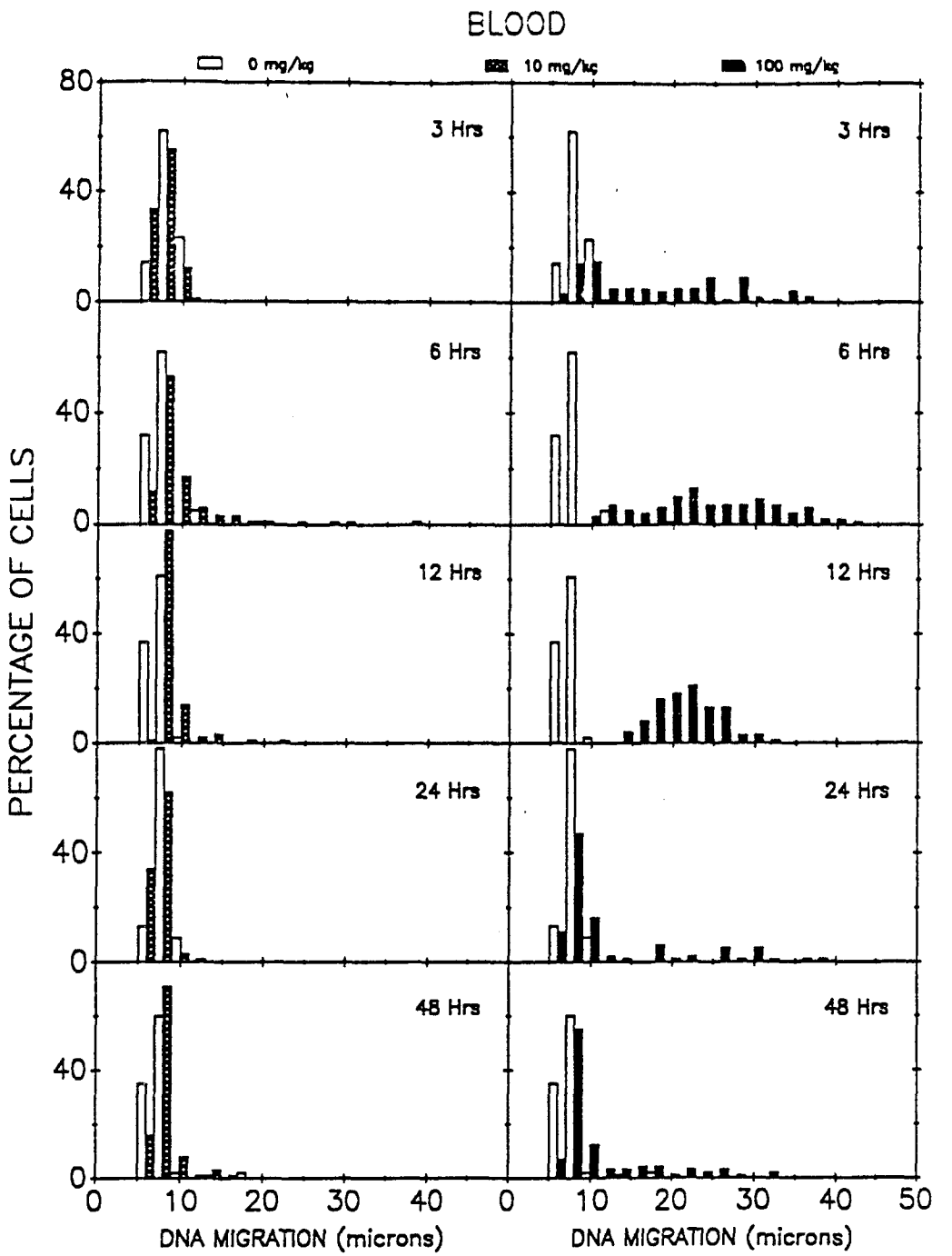

Figure 37. Sample Time Dependent Distribution of DNA Migration Lengths for Mouse Blood Leukocytes Collected from Acrylamide-Treated Male B6C3F1 Mice. Data based on 100 cells per sample. The width of each bar represents 2 microns.

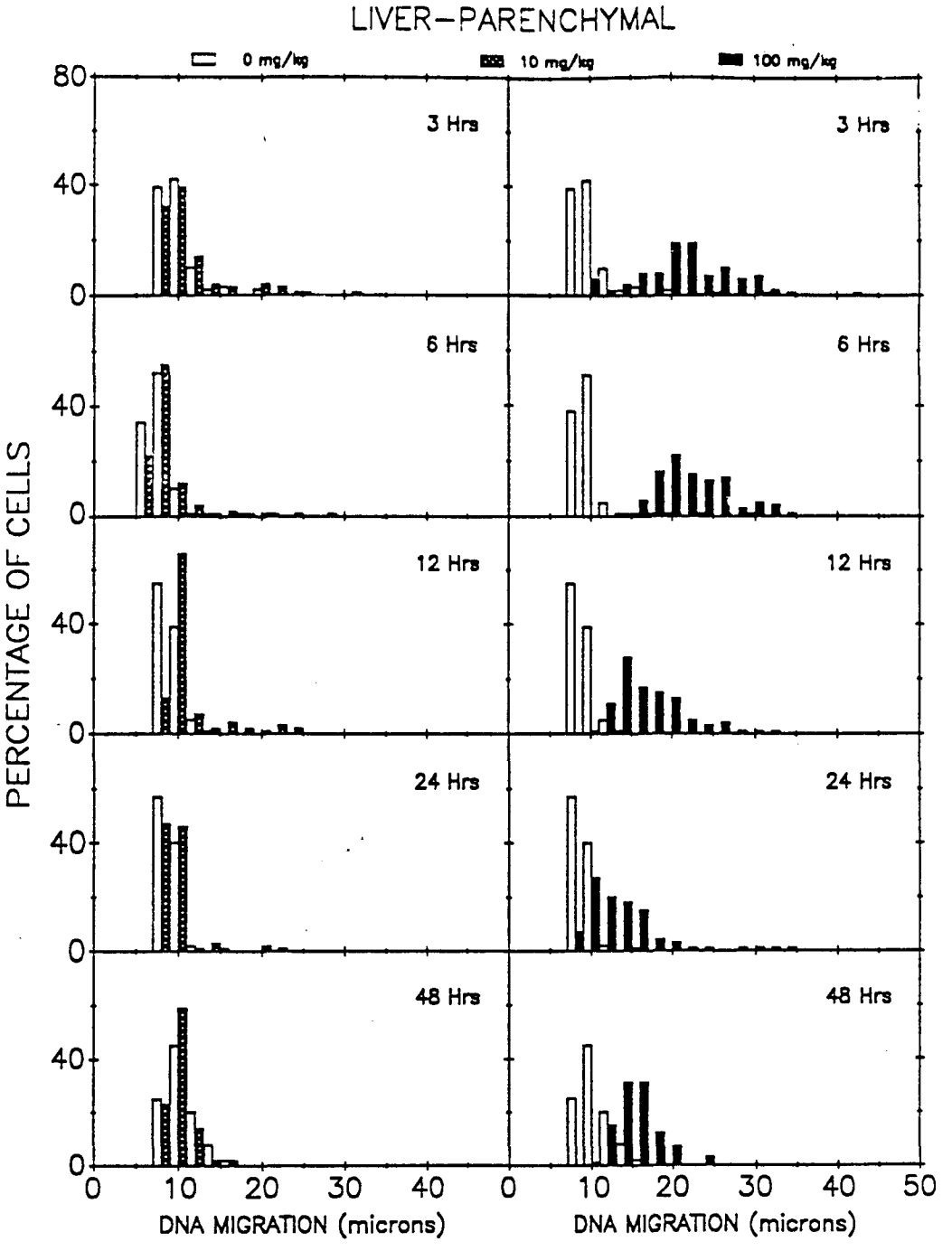

Figure 38. Sample Time Dependent Distribution of DNA Migration Lengths for Mouse Liver Parenchymal Cells Collected from Acrylamide-Treated Male B6C3F1 Mice. Data based on 100 cells per sample. The width of each bar represents 2 microns.

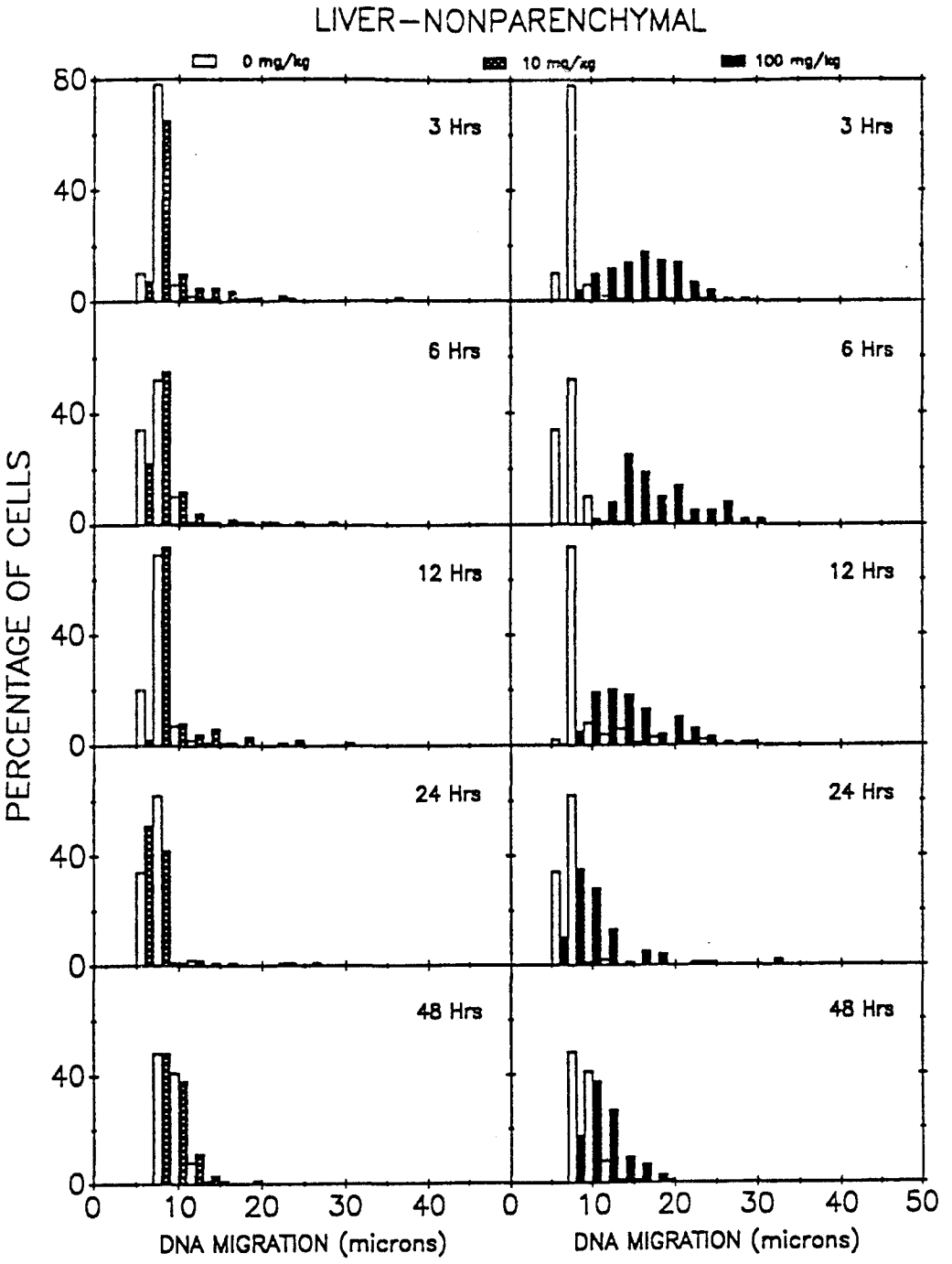

Figure 39. Sample Time Dependent Distribution of DNA Migration Lengths for Mouse Liver Nonparenchymal Cells Collected from Acrylamide-Treated Male B6C3F1 Mice. Data based on 100 cells per sample. The width of each bar presents 2 microns.

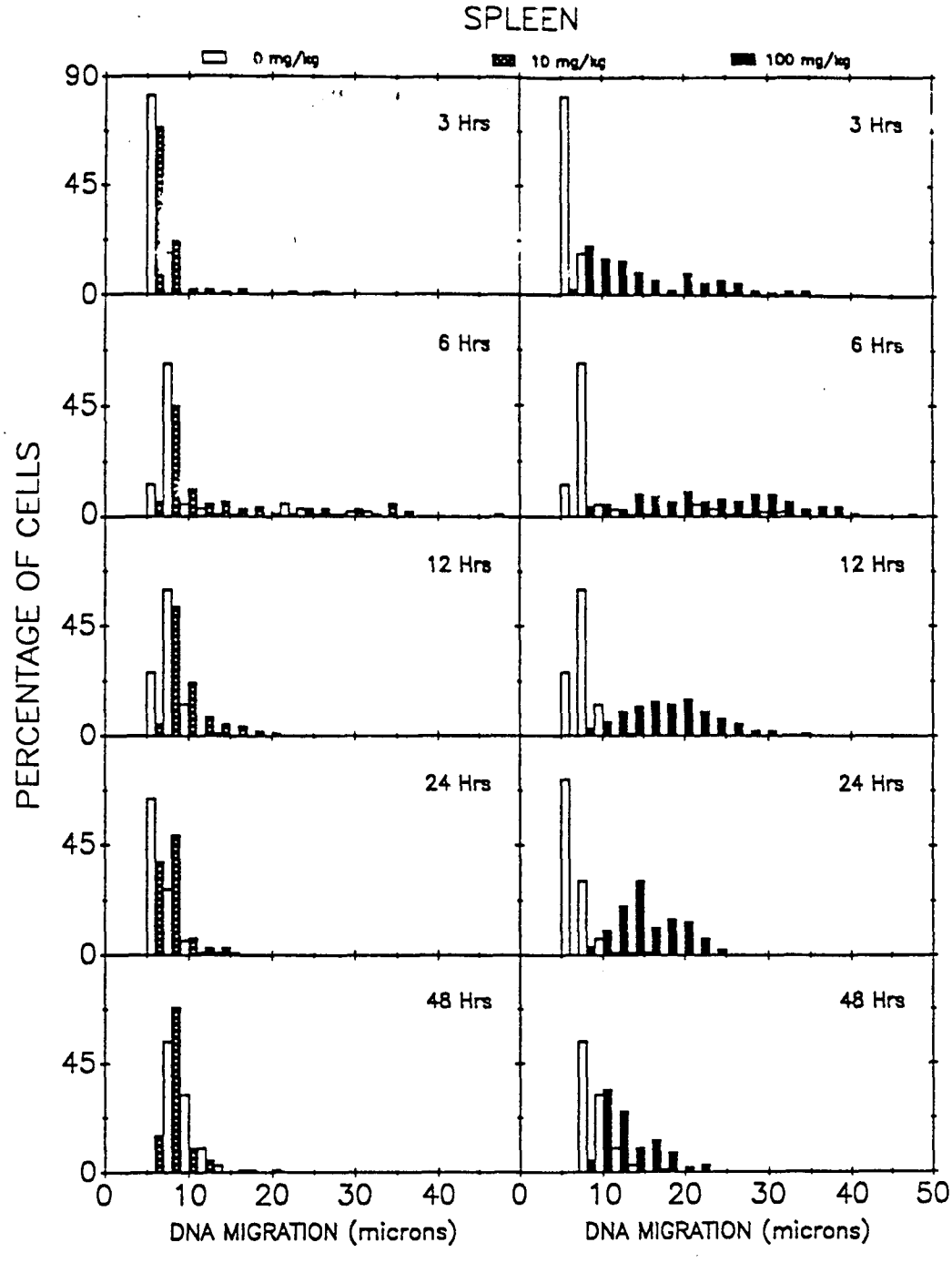

Figure 40. Sample Time Dependent Distribution of DNA Migration Lengths for Mouse Spleen Cells Collected from Acrylamide-Treated Male B6C3F1 Mice. Data based on 100 cells per sample. The width of each bar represents 2 microns.

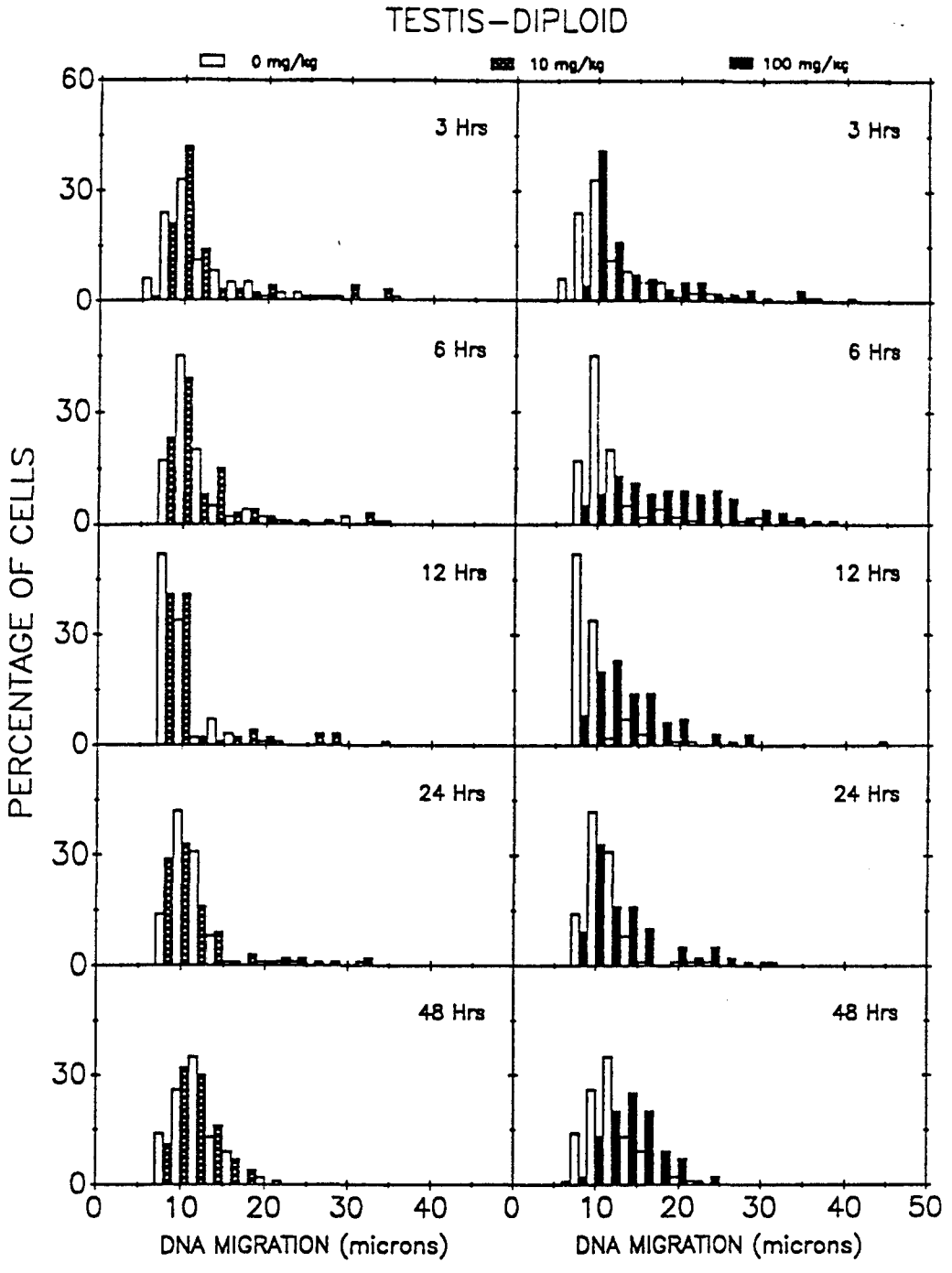

Figure 41. Sample Time Dependent Distribution of DNA Migration Lengths for Mouse Testis Diploid Cells Collected from Acrylamide-Treated Male B6C3F1 Mice. Data based on 100 cells per sample. The width of each bar represents 2 microns.

conducted and/or are in progress to examine the utility of the SCG assay in human biomarker studies.

7.1 5K Race Study

In a very early study, peripheral blood was sampled from three individuals participating in a 5K road race. While this study may be perceived as being unusual, it was felt that obtaining samples under these conditions would demonstrate the feasibility of conducting similar studies outside the laboratory. Three runners were involved and samples were obtained by finger prick prior to the start of the race (within 5 to 15 minutes), within 5 minutes after completion of the course, and at one-half and one hour later. The samples were kept cold, transported back to the laboratory approximately 2 hours after the initial sample and processed in the SCG assay. Two of the runners (runners 1 and 3) exhibited no increase in DNA migration immediately after the completion of the race, while one runner (runner 2) exhibited a large increase in DNA migration. While conducted under less than rigorous conditions and before the affect of various subpopulations of leukocytes in the peripheral blood on DNA migration patterns was fully appreciated, the study does indicate the feasibility of utilizing this technique for conducting human studies outside the laboratory.

7.2 Smokers vs Nonsmokers

In another, early study, peripheral blood was obtained from three heavy smokers and three, age and sex-matched control nonsmokers. A blood sample, obtained by finger prick, was obtained early in the morning after each smoker had completed one cigarette. After being processed in the SCG assay, with samples being electrophoresed for either 20 or 30 minutes, DNA migration in 50 cells for each individual was determined. At 20 minutes of electrophoresis, the extent of DNA migration was not different between the smokers and nonsmokers (Figure 42). At 30 minutes of electrophoresis, the DNA migration patterns among the smokers was less than that observed for the nonsmokers (Figure 42). This was due to the presence of a larger population of leukocytes among the blood from the nonsmokers with extended DNA migration patterns. Several hypothesis for this surprising difference were considered. First, we considered that the differences was due to increased levels of DNA crosslinking among the cells from the smokers. Based on hypothetical grounds, increased crosslinking would result in decreased migration. Second, we considered the possibility that the difference in migration may reflect differences in lymphocyte population dynamics/turnover between the two groups. Finally, it was suggested that smokers may have induced levels of DNA repair enzymes; thus lower levels of background damage.

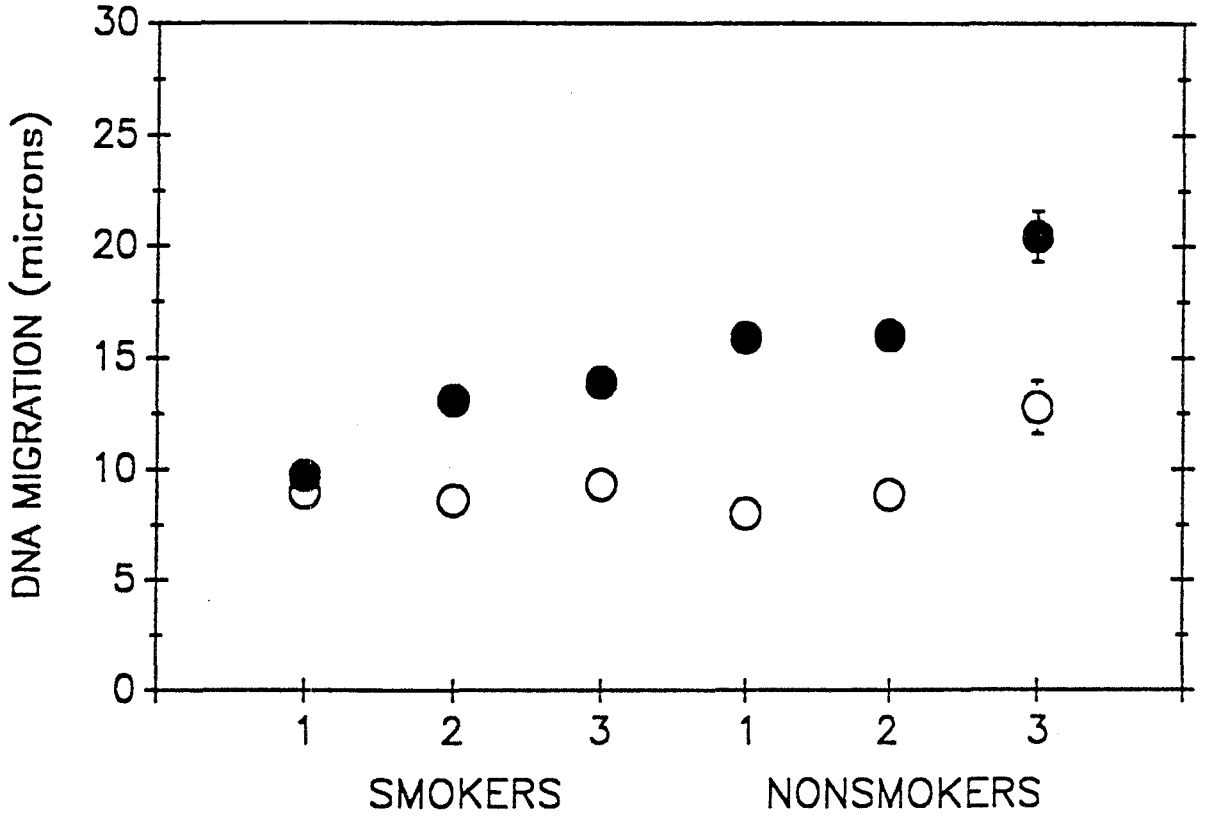

Figure 42. DNA Migration Lengths for Blood Leukocytes Sampled from Three Smokers and Three Nonsmokers (Experiment 1). Electrophoresis was for either 20 (open symbols) or 30 (closed symbols) minutes. Each symbol represents the mean and standard error of the mean for 50 cells.

Based on observations made on blood samples stored in the laboratory for several days, a second smokers study was subsequently conducted in which DNA migration patterns were compared for fresh blood or for blood stored for 48 hours. In both cases, the DNA was electrophoresed for 20 or 40 minutes. The results of this experiment are presented in Figure 43. Again, no difference in migration was detected in fresh blood electrophoresed for 20 min. Again, except for one smoker with elevated migration lengths, the DNA from the nonsmokers appeared to migrate more at 40 minutes of electrophoresis than the DNA from the smokers. However, when the blood was stored for 48 hours and then run on the SCG assay, DNA migration patterns were not different between smokers and nonsmokers at either electrophoresis duration. In fact, it appeared as if the cells with the extended migration patterns had disappeared during this incubation period from each individual's blood (Figure 44). This resulted in a population of cells with almost a complete lack of DNA migration. We subsequently determined, using hydrogen peroxide treated cells, that damage, in and of itself, did not lead to a disappearance of damaged cells from whole blood over this period of time. Rather, we speculate, that a subpopulation of cells, probably neutrophils, are either depressed in number or in capability, in the blood from smokers. Experiments to determine if this hypothesis is correct are planned.

7.3 Duke Cancer Study

In this study we attempt to ascertain the utility of the (SCG assay in evaluating DNA damage in peripheral blood leukocytes from individuals being treated with high-dose alkylating agents. Patients studied were under the care of the Bone Marrow Transplant Program at Duke University Medical Center. Twelve patients with metastatic breast tumors were sampled before, during and after the intravenous (IV) administration of antineoplastic alkylating agents. The patients received indwelling, triple lumen, right atrial catheters for venous access prior to treatment. The transplant preparative regimen was as follows: cyclophosphamide (CTX) was administered as a 1 h infusion on days -6,-5,-4 at a dose of 1,875 mg/m^2/d; cisplatin (cis-DDP) (165 mg/m^2) was given with hydration by continuous infusion over 72 h from day -6 to -3; carmustine (600 mg/m^2) was infused on day -3 at a rate of 5 mg/m^2/min. On day 1 (3 days following completion of chemotherapy) autologous bone marrow, harvested and cryopreserved prior to drug treatments, was thawed and rapidly infused. Blood specimens for the SCG study were taken as follows: on day -6, before drug treatment; on day -4, following 2 days administration of CTX and cis-DDP and upon hematopoietic recovery (when peripheral blood white cell counts increase to at least 1,000/m^3.

PBLs were isolated from heparinized blood using Lymphocyte Separation Medium (LSM) (Organon Teknika-Cappel, Durham, NC).

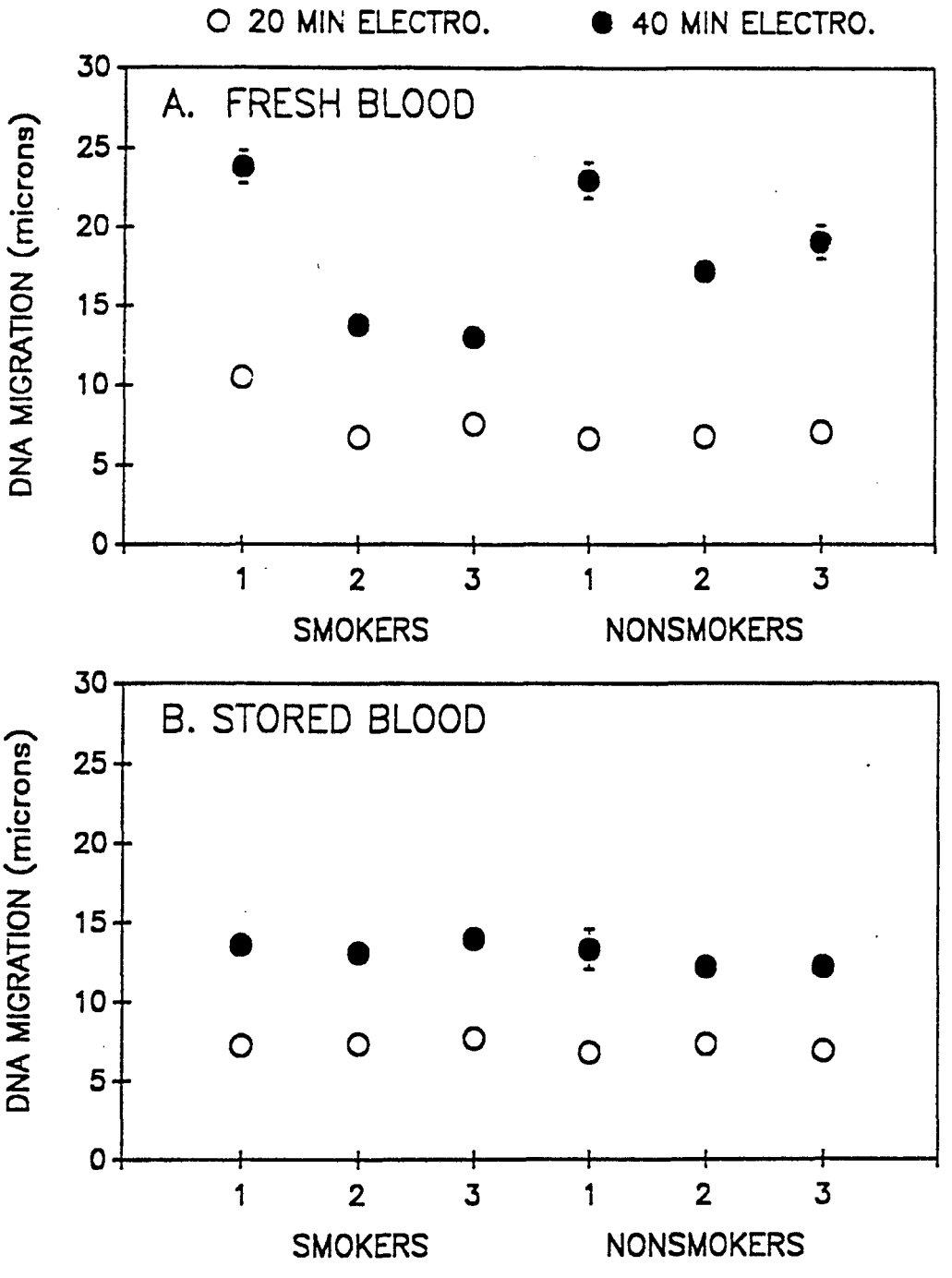

Figure 43. DNA Migration Lengths for Blood Leukocytes Sampled from Three Smokers and Three Nonsmokers (Experiment 2). Electrophoresis was for either 20 or 40 minutes. Electrophoresis was for either 20 (open symbols) or 40 (closed symbols) minutes. Each symbol represents the mean and standard error of the mean for 50 cells.

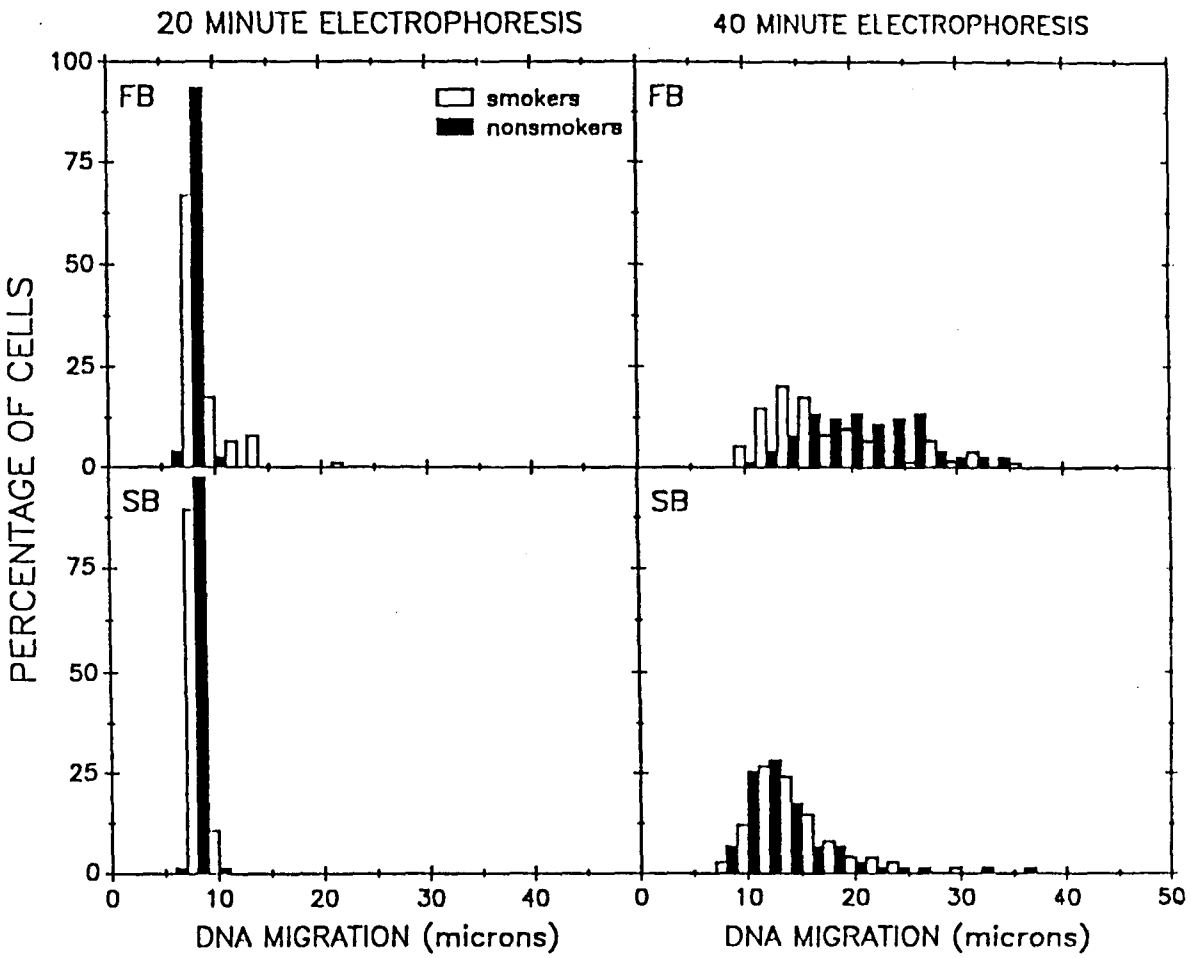

Figure 44. Distribution of DNA Migration Lengths for Blood Leukocytes Sampled from Three Smokers and Three Nonsmokers (Experiment 2). Data based on 50 cells per sample. The width of each bar represents 2 microns. FB = fresh blood; SB = stored blood.

Briefly, 6 - 7 ml blood were carefully layered over 4 ml LSM and centrifuged at 400 x g for 20 minutes. The buffy layer was removed, and the viable lymphocytes and monocytes counted using 1.0% trypan blue (Sigma). The plasma was saved for use in further cell handling. The cells were washed with TC-199 medium (Gibco) by centrifugation at 400 x g for 10 minutes and aspiration of the supernatant. Cells to be assayed fresh were resuspended at approximately 10^7/ml in medium with 20% plasma. Media TC-199 and plasma, at final proportions of 70% and 20% respectively, were added to resuspend the cell pellet. DMSO (Sigma) was then added at 10% (v/v) and the cell suspensions were transferred to plastic freezing vials (NUNC) in 1 ml aliquots containing approximately 10^7 cells each. Vials were placed directly into a -70°C freezer. As needed, vials were retrieved and placed in a 37°C water bath and shaken gently until melting of the last ice crystal.

For SCG analysis, 5-10 ul samples were mixed with low melting agarose and processed as described (electrophoresis was for 20 minutes). For each sample, 25 cells were scored on each of two slides (50 cells total) for DNA migration. The mean DNA migration length for each sample analyzed to date are presented in Figure 45, with the corresponding histogram data presented in Figure 46 and 47. Clearly, there are differences among the individuals in regard to either the level of DNA damage present in the admission sample, the sample obtained after two days of chemotherapy, and in the sample obtained after hematopoietic recovery. The significance of these differences in terms of various pretreatment regimens, the chemotherapy outcome, and or various in vitro bone marrow treatments requires additional information, both about the patients and about the relative ability of the various chemotherapy drugs to induce DNA damage, as measured by the SCG assay. It is unfortunate that samples are not available after the last day of treatment. The lack of this sample makes any correlation with therapy outcome more difficult to interpret. However, the data collected demonstrate the potential utility of the SCG assay in human studies. Additional samples, including both fresh and frozen blood samples from the same patient continue to be analyzed for DNA damage.

7.4 Human Longitudinal Study

Very early in the course of this project we decided to conduct a longitudinal study to evaluate the extent of interindividual variability in DNA migration in human leukocytes. While we recognized that such a study would be labor-intensive, we believed that the resulting data would be important in any human monitoring study. A blood sample was obtained by fingerprick from a group of volunteers consisting of three males and three females. One male smoker and one female smoker was included in the group. Blood samples were obtained generally each week (in some cases every other week) over a period of approximately 6

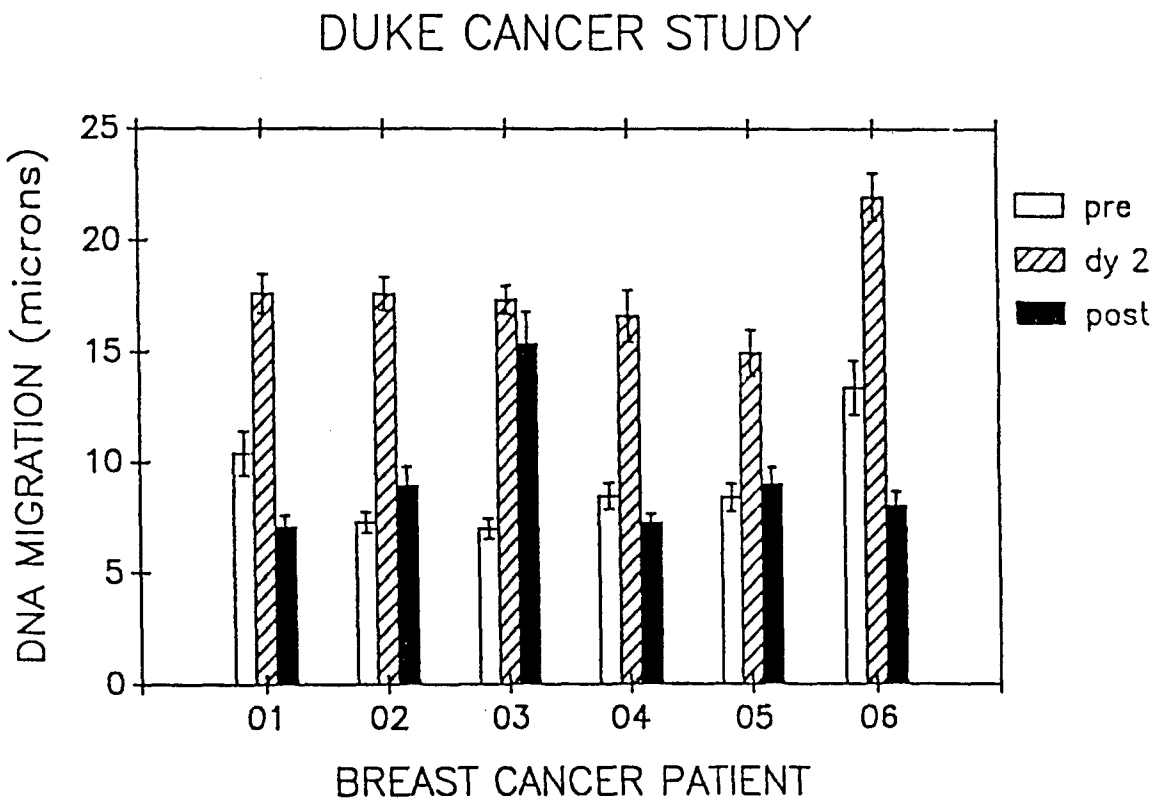

Figure 45. DNA Migration Lengths for Blood Leukocytes Sampled from 6 Duke Hospital Chemotherapy Patients. Samples were obtained upon admission (pre), after 2 days of a 3-day treatment protocol (dy 2), or after hematopoietic recovery (post). Electrophoresis was for 20 minutes. Each bar indicates the mean and standard error of the mean for 50 cells per sample.

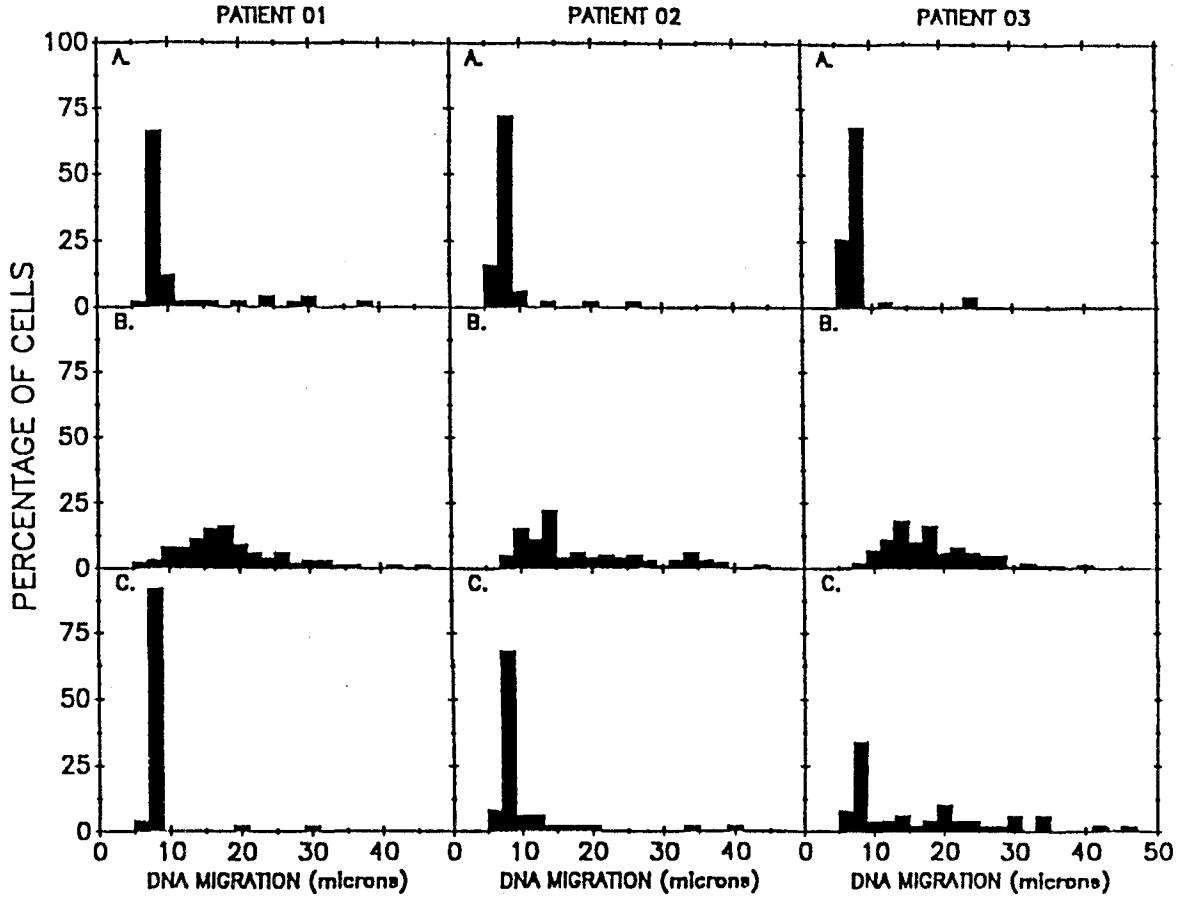

Figure 46. Distribution of DNA Migration Lengths for Blood Leukocytes Sampled from First 3 Duke Hospital Chemotherapy Patients. Samples were obtained upon admission (A), after 2 days of a 3-day treatment protocol (B), or after hematopoietic recovery (C). Electrophoresis was for 20 minutes. Data based on 50 cells scored per sample.

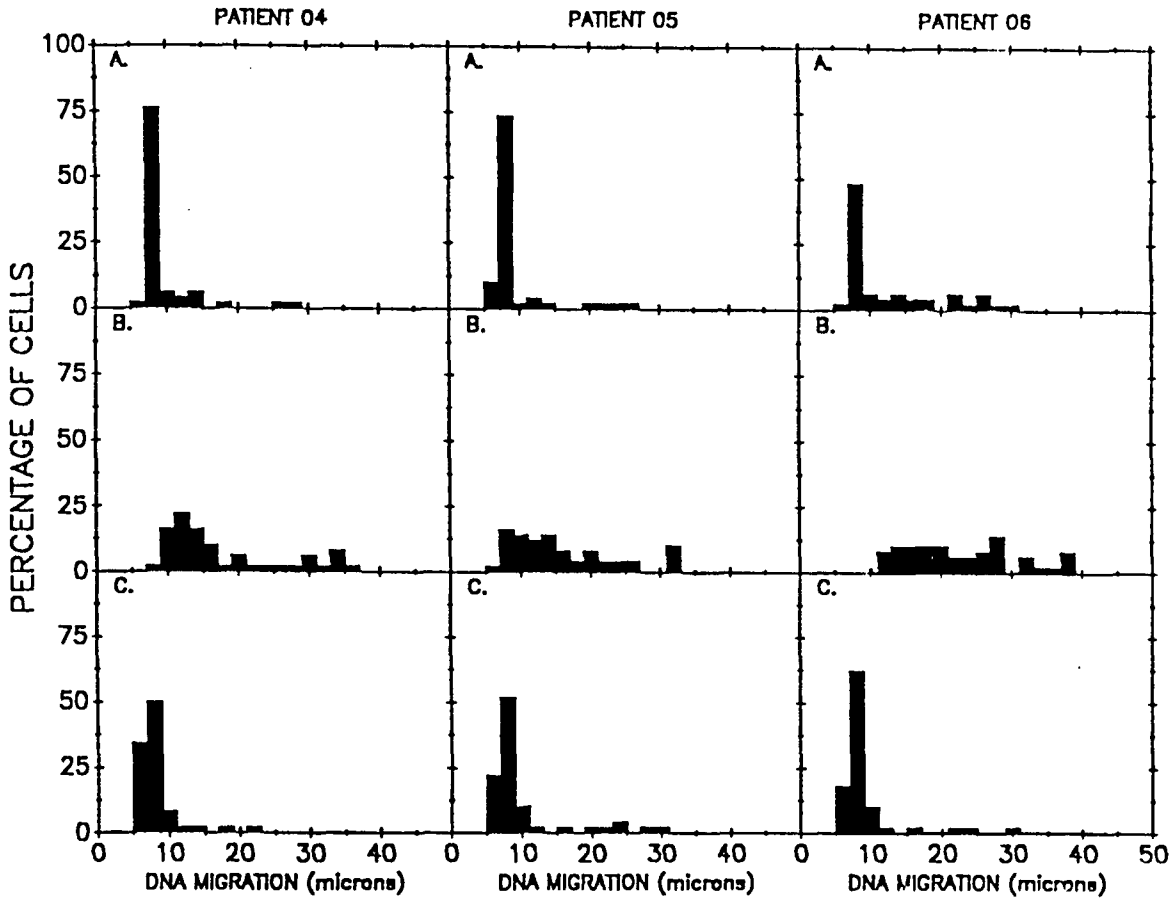

Figure 47. Distribution of DNA Migration Lengths for Blood Leukocytes Sampled from Second 3 Duke Hospital Chemotherapy Patients. Samples were obtained upon admission (A), after 2 days of a 3-day treatment protocol (B), or after hematopoietic recovery (C). Electrophoresis was for 20 minutes. Data based on 50 cells scored per sample.

months. Duplicate slides were prepared, with one slide being electrophoresed for 20 minutes and the other for 40 minutes. Fifty cells were scored per slide. Many of the technical problems already discussed were detected in this on-going study, with the loss of a significant number of data points. Furthermore, when the results of the smoker and blood storage experiments indicated that significant variability in DNA migration in fresh whole blood samples could be largely due to leukocyte subpopulation shifts, we decided to stop this study and discard the data. We still recognize the need of such a study, but several kinds of experiments must be conducted before the results could be interpreted correctly.

8.0 HAZARDOUS WASTE SITE STUDIES

The ultimate goal of this research project was to evaluate the potential of the SCG assay for detecting DNA damage in free-living rodents inhabitating a hazardous waste site. It was hypothesized that such animals would express increased levels of DNA damage and that information on the organ-distribution, persistence and magnitude of such damage could be used in evaluating the potential for ill-health effects in near-by human communities. Clearly, at the present time, while the extrapolation of such data to human health effects is speculative, information on the presence or absence of increased levels of DNA damage in such animal populations would be useful in categorizing different sites and in indicating the need for appropriate human studies. Techniques, such as the SCG assay, to evaluate global levels of DNA damage, would play an important role in such studies, especially if they could be applied to similar tissues in human populations.

8.1 Study Area

Because of difficulties in obtaining permission to gain access to EPA superfund sites, we decided to collect rodents at the perimeter of a near-by site. This study was conducted at the North Carolina State University Hazardous Waste (HW) Facility (NCSU Lot #86, Farm Unit #1) located approximately 0.5 miles west of the intersection of Blue Ridge Rd. and West Chase Dr., Raleigh, NC (Fig. 48). The site is situated in the Lower Piedmont, a plateau of gently rolling hills (Hurst, 1963), with an elevation of 450 ft. The study site is occupied by the Appling soil association, which is characterized by moderately sloping to strongly sloping acidic soils of Piedmont uplands. The surface layer is composed of either gravelly sandy loam, sandy loam, or fine sandy loam with a subsoil of firm clay loam to clay (Cawthorn, 1970). The site has been classified as a Superfund site, with the predominant pollutants including TCE, chloroform, carbon tetrachloride, various pesticides, laboratory solvents, and other chemicals. Although the selected site is

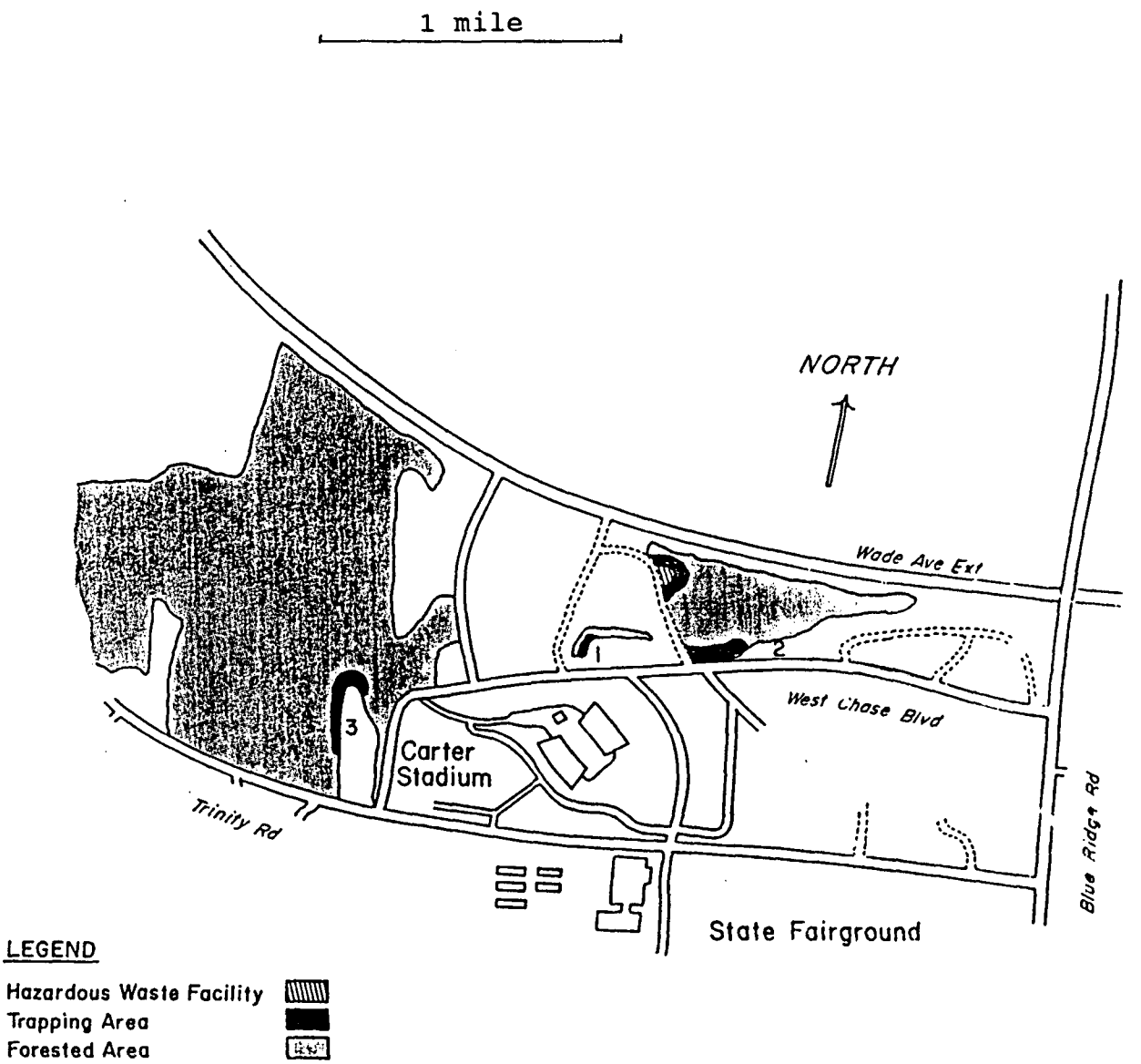

Figure 48. Location of the North Carolina State University EPA Superfund Site.

quite small, the level of exposure anticipated to be quite limited, and the numbers of animals few, we believed that this study would be useful in determining the potential applicability of the SCG assay to such field studies.

The HW site is a fenced 1.5 acre grassy area surrounded on three sides by a pine-hardwood forest and on one side by a dirt road (Fig. 49). Three control sites were used during this study, and the reason for each will be addressed below. Control sites #1 and #2 (Fig. 48) were located approximately 600 ft southwest and southeast, respectively, of the HW site. Control site #3 was located approximately 1400 ft southwest of the HW site. The HW site and control site #2 were located within the same pine-hardwood forest. Dominant vegetation included Pinus taeda (loblolly pine), P. virginiana (Virginia pine), Quercus alba (white oak), Q. marilandica (blackjack oak), Liquidambar styraciflua (sweetgum), Acer rubrum (red maple), Cornus florida (flowering dogwood), Rubus allegheniensis (blackberry), Rosa spp. (roses), Lonicera japonica (Japanese honeysuckle), and Rhus toxicodendron (poison ivy). Pine needles covered the forest floor, and Pueraria spp. (Kudzu-vine) formed the ground cover on the first half of the HW study area along the northern fence line. Control site #1 was a narrow band of P. taeda, Q. alba, L. styraciflua, C. florida, R. allegheniensis, and Rosa spp. surrounded by an unmowed field. Control site #3 was a pine-hardwood forest surrounded by an unmowed field. Dominant vegetation included P. taeda, Q. alba, Liriodendron tulipifera (tulip poplar), L. styraciflua, A. rubrum, R. allegheniensis, Rosa spp., L. japonica, R. toxicodendron, Smilax rotundifolia (common greenbrier), and S. walteri (redberry greenbrier). Pine needles covered the forest floor.

8.2 Live-Trapping Schedule

Ochrotomys nuttalli (Golden mouse) were live-trapped during 6 trapping sessions from 21 May, 1990, to 13 June, 1990. Thirty-five Sherman traps each were placed along the outside perimeter of 2 fenced sides of the HW site and on a control site (Fig. 49). Traps were set and baited (rolled oats) in late afternoon and checked at sunrise the following morning. Captured animals were identified by species, and their sex and reproductive condition were recorded. All O. nuttalli, excluding lactating females, were then returned to their respective traps while other species caught (Blarina carolinensis (Southern short-tailed shrew), Tamias striatus (Eastern chipmunk), and Sigmodon hispidus (hispid cotton rat)) were released. Animals were transported to the lab in traps.

The control site was moved after the first trapping session because of a difference in species predominance: S. hispidus on the control site and O. nuttalli on the HW site. The second control site capture results were similar to those of the HW Fig

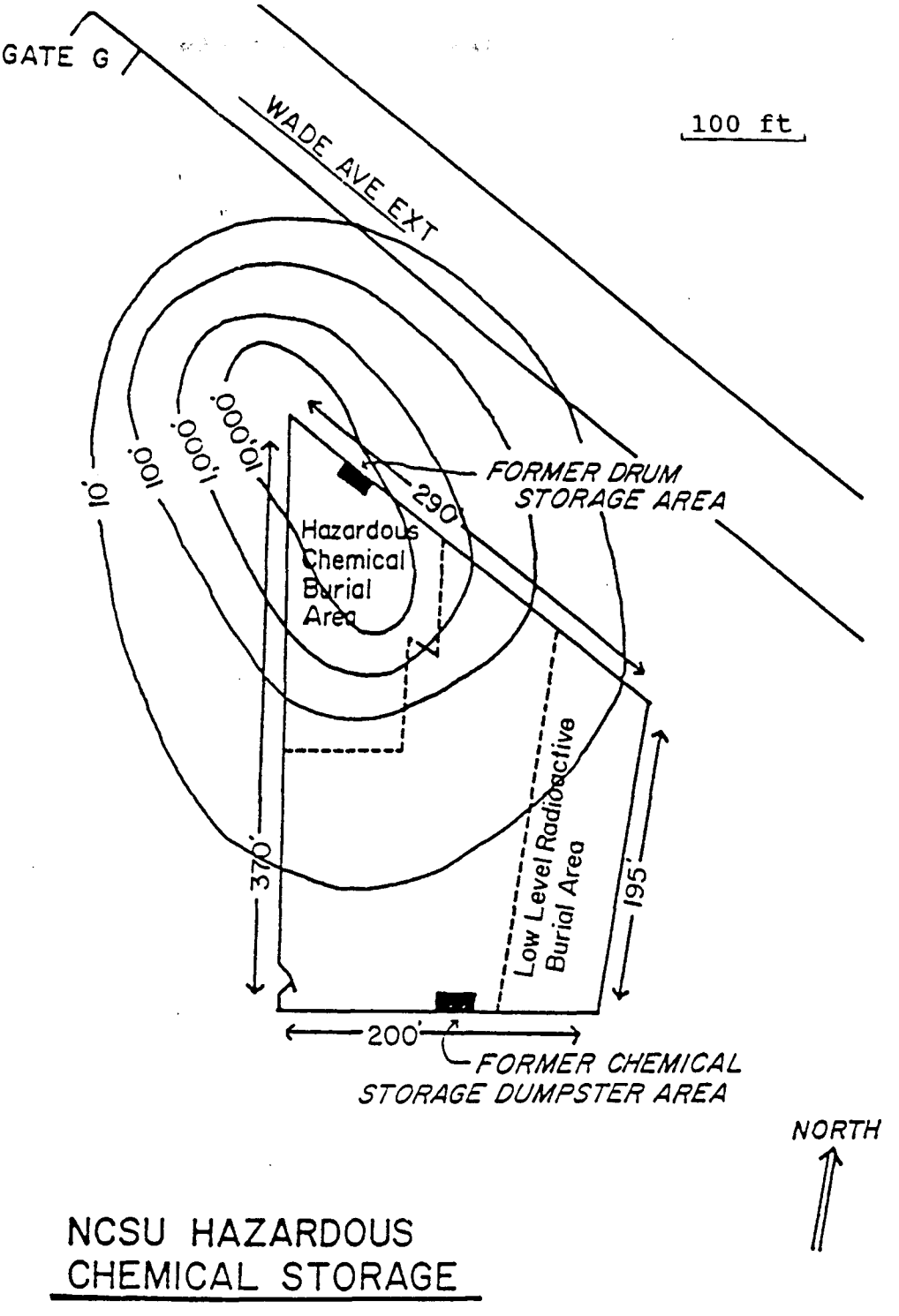

Figure 49. An expanded view of the North Carolina State University Superfund Site. The isopleths indicate concentrationz (ug/l) of tetrachloroethene, chloroform and carbon tetrachloride contamination in the ground water.

site. After the second trapping session, traps on the HW site were placed along a different fenced side in an area of tetrachloroethene, chloroform, and carbon tetrachloride concentration plumes (Fig. 49). After the third trapping session, 15 traps were moved to the control site to increase capture success. Twenty traps from the control site were stolen after the fourth trapping session. As a result, a new control site with 60 traps was established for the sixth trapping session, and trapping on the HW site was terminated.

8.3 Tissue Collection

O. nuttalli were sacrificed via CO_2 asphyxiation, and their body weight, body length, and tail length were recorded. Subsequently, peripheral blood, liver, brain and bone marrow samples were obtained from each animal and evaluated for DNA damage using the SCG technique. In addition, bone marrow and peripheral blood smears were prepared on each animal to evaluate micronuclei (MN) frequencies in polychromatic erythrocytes (PCE) and the percentage of PCE among total erythrocytes (a measure of the rate of erythropoiesis).

8.4 Data Analysis

A total of 13 mice each were collected at the HW site and among the various control sites. Population demographics were comparable between the two populations of animals (Table 1). An initial inspection of MN frequencies in PCE in peripheral blood indicated that the efficiency of this rodent's spleen in removing micronucleated PCE from the circulating blood eliminated the possibility of evaluating PCE in these smears. In bone marrow smears, 1000 PCE per animal were scored for the presence of MN and the number of PCE among 200 erythrocytes was determined using an acridine orange staining technique. No difference between the HW and control groups in either the frequency of MN-PCE or the percentage of PCE were observed (Table 2).

The selection of the various tissues for an evaluation of DNA damage was based on the following premises. First, liver was selected as the organ largely responsible for the metabolic activation and detoxification of xenobiotics. Furthermore, since the cells in this organ are largely terminally-differentiated it might be anticipated that DNA damage would accumulate throughout the life-time of the animal. Brain was selected because the cells of this organ are also terminally differentiated, but lack many of the protective mechanism inherent to liver cells. Thus, DNA damage may be expected to accumulate at a greater rate. Blood was selected based on our limited in vivo laboratory animal experience indicating that blood leukocytes appear relatively more deficient in repair than liver, spleen, or testis and because blood is the tissue most readily obtainable from human populations. Finally, bone marrow was selected because of the

TABLE 1. POPULATION DEMOGRAPHICS OF *Ochrotomys nuttalli* TRAPPED ON THE CONTROL AND HW SITES OF THE NCSU HW FACILITY.

ANIMAL NUMBER	TRAP I.D.	AGE	SEX	REPROD. COND.	BODY WT.(g)	TOTAL LENGTH(mm)	TAIL LENGTH(mm)
01	H13	JUV	M	SCROTAL	14.5	133.350	57.150
02	H26	JUV	F	NON-EST	12.2	136.520	60.325
03	H25	SA	M	NON-SCR	16.3	146.050	63.500
04	H02	SA	M	NON-SCR	15.2	158.750	76.200
05	H29	SA	F	ESTROUS	15.6	155.575	69.850
06	H06	AD	M	NON-SCR	18.1	158.750	69.850
07	C04	SA	M	SCROTAL	18.2	139.700	57.150
08	C05	JUV	F	NON-EST	13.0	133.350	57.150
09	C16	SA	F	NON-EST	16.6	139.700	57.150
10	C31	SA	F	NON-EST	14.7	142.875	63.500
11	H08	JUV	M	NON-SCR	14.0	139.700	63.500
12	C10	JUV	M	NON-SCR	13.7	136.525	63.500
13	C13	SA	F	NON-EST	18.7	152.400	69.850
14	H08	JUV	M	NON-SCR	14.2	139.700	57.150
15	H11	JUV	M	NON-SCR	16.9	130.175	50.800
16	H17	JUV	F	NON-EST	14.7	139.700	60.325
17	C27	JUV	F	NON-EST	11.0	120.650	50.800
18	H11	JUV	F	NON-EST	12.7	139.700	63.500
19	C36	JUV	M	NON-SCR	14.6	136.525	57.150
20	C48	SA	F	NON-EST	17.6	149.225	66.675
21	H03	JUV	F	NON-EST	11.8	114.300	50.800
22	H20	AD	M	SCROTAL	21.4	152.400	63.500
23	C52	JUV	M	NON-SCR	11.5	123.825	57.150
24	C51	JUV	M	NON-SCR	11.9	130.175	57.150
25	C50	JUV	M	NON-SCR	14.3	139.700	63.500
26	C20	AD	M	NON-SCR	21.2	158.750	73.025

Codes: H = HW site; C = Control Site; AD = Adult; JUV = Juvenile; SA = Subadult; NON-EST = Non-estrous; NON-SCR = Non-scrotal

Table 2. Frequencies of Micronucleated Polychromatic Erythrocytes and the Percentage of Polychromatic Erythrocytes in Bone Marrown of Animals Collected at the North Carolina State Univeristy Hazardous Waste Site and the Concurrent Control Sites.

Hazardous Waste				Concurrent Control			
Animal Number	Sex	MN-PCE/ 1000 PCE	%PCE	Animal Number	Sex	MN-PCE/ 1000 PCE	%PCE
01	m	3	54.0	07	m	4	44.0
02	f	3	47.0	08	f	3	82.0
03	m	2	51.0	09	f	3	71.0
04	m	3	53.0	10	f	2	57.0
05	f	2	35.0	12	m	10	51.0
06	m	4	53.0	13	f	4	48.0
11	m	2	37.0	17	f	5	35.0
14	m	10	66.0	19	m	5	70.0
15	m	3	74.0	20	f	1	40.0
16	f	4	61.0	23	m	4	60.0
18	f	5	55.0	24	m	4	67.0
21	f	4	62.0	25	m	6	52.0
22	m	8	26.0	26	m	5	48.0
Group Mean		4.08	51.8			4.31	55.8
sem		0.66	3.7			0.60	3.8
P Value		MN-PCE = 0.6133				%PCE = 0.2382	

P-value from one-tailed pairwise comparison based on liklihood ratio using pooled data.

opportunity to compare SCG data with another endpoint (i.e., MN) and because DNA damage in this tissue would be more indicative of a recent exposure to genotoxicants, either through increased body burdens of reactive pollutants or through concurrent environmental exposures.

The group mean DNA migration length and dispersion data are presented in Figure 50. In addition, for a better inspection of individual animal responses, the data for animals within each group, by tissue, were ranked from low to high and plotted individually (Figure 51, 52). Not surprisingly, the extent of interanimal variability was much greater than that observed normally for laboratory animals. The level of DNA damage, as measured by mean migration length, was increased in all four tissues of animals trapped near the HW site, but only significantly in brain ($P = <0.05$). However, a dispersion analysis revealed that the bone marrow cells from the HW mice exhibited a significantly increased dispersion coefficient over that calculated for the control mice ($P = <0.05$). This increased dispersion was due to small numbers of cells with extended DNA migration patterns among a majority of cells with no or little DNA migration. This result suggests the presence of low levels of genotoxic species in this organ and/or differential sensitivity among the various subpopulations of cells that comprise this tissue. An example of the distributional data for DNA migration obtained on a representative control and HW mouse is presented in Figure 53. An analysis was conducted to evaluate the degree of correlation among tissues within each animal in the extent of DNA migration or dispersion. The only significant correlation occurred for the dispersion of DNA migration in brain and liver cells (Figure 54). The absence of a significant tissue correlation suggests that the increases in DNA damage result from independent events. Trap location, sex or age-dependent differences in response were not present, due most likely to the limited number of animals collected.

The results of this small, pilot study indicate the potential usefulness of the SCG technique in evaluating DNA damage in free-living rodents. Without knowing the numerically prevalent mammal on this site, enough individuals were collected that a reasonable and informative analysis could be conducted. Clearly, considerable more work is needed to fully characterize the limitations and capabilities of the SCG technique in this system. In any study, the possible influences of animal health, food resources, etc, on SCG data must be considered when evaluating the resulting data. For example, increased levels of brain cell damage may result from food practices or from parasitic infections. Also, it would be extremely useful if body burdens of various pollutants are evaluated concurrently in the same animals.

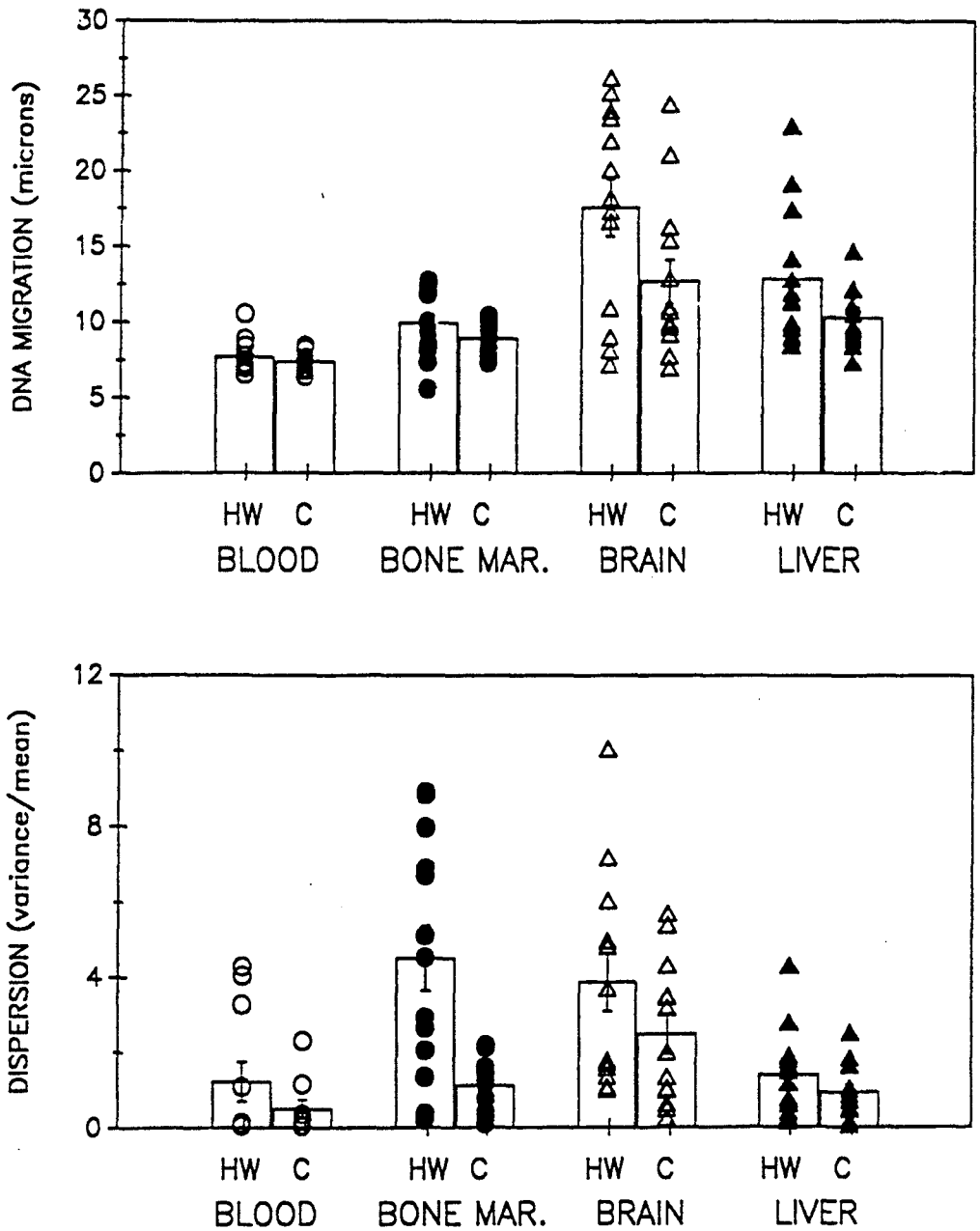

Figure 50. Evaluation of DNA Damage in Various Tissue of O. nuttalli Collected at the North Carolina State University EPA Superfund Sites as Compared to Animals Collected from Concurrent Control Sites. Bar heights represent group means, error bars indicate the standard error of the mean among animals (13 for each group except for blood which involved 11 hazardous waste mice and 9 control mice). Each symbols represents an individual animal response. 25 cells were scored per tissue.

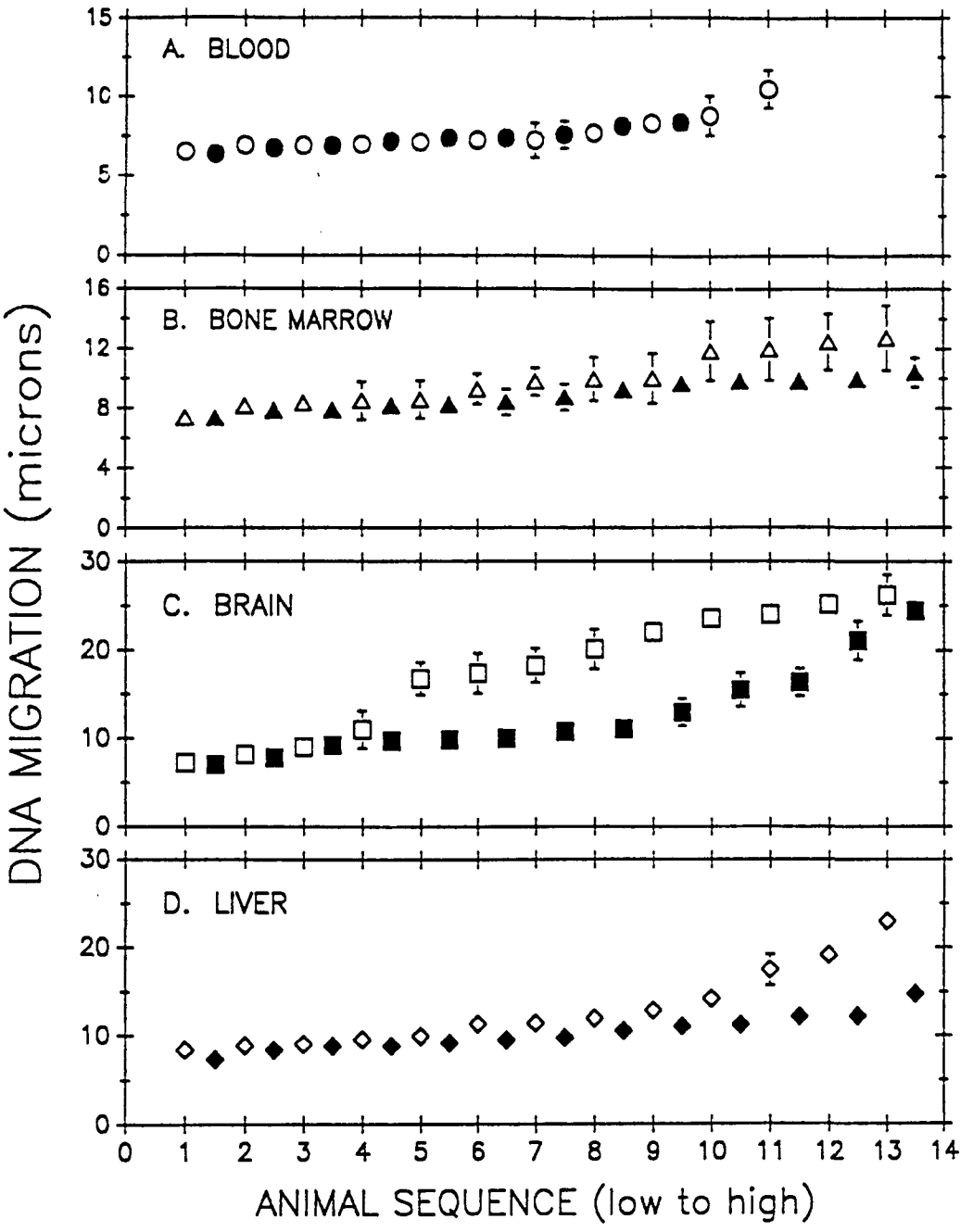

Figure 51. A Plot of Individual Animal Responses for DNA Migration. The data are ranked in order from low to high DNA migration length, alternating between hazardous waste and control mice. Each symbol represents the mean and standard error of the mean among 25 cells. Open symbols indicate hazardous waste mice, solid symbols represent control mice.

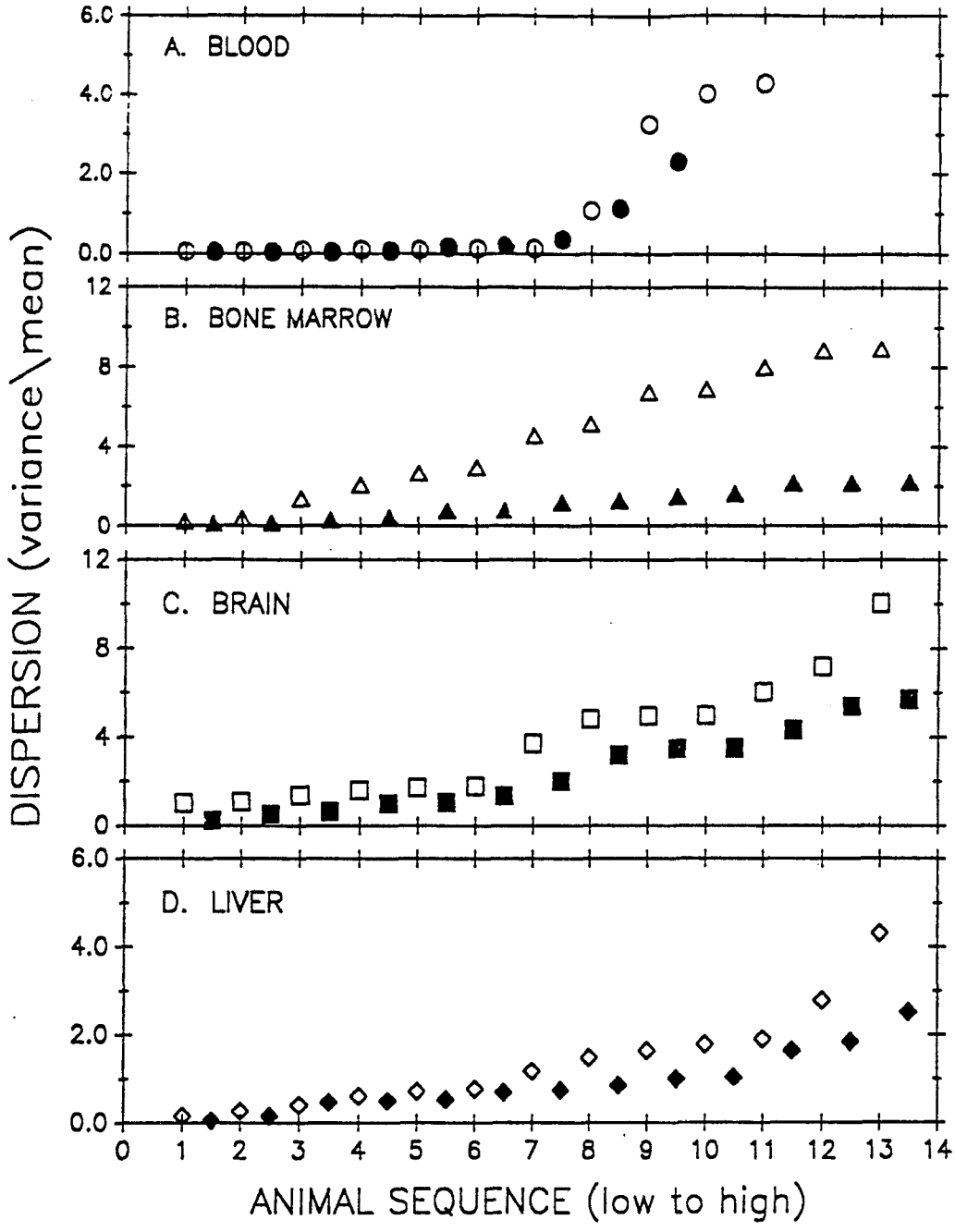

Figure 52. A Plot of Individual Animal Responses for Dispersion Coefficient. The data are ranked in order from low to high dispersion coefficients, alternating between hazardous waste and control mice. Each symbol represents the dispersion coefficient (H = variance/mean) of 25 cells. Open symbols indicate hazardous waste mice, solid symbols represent control mice.

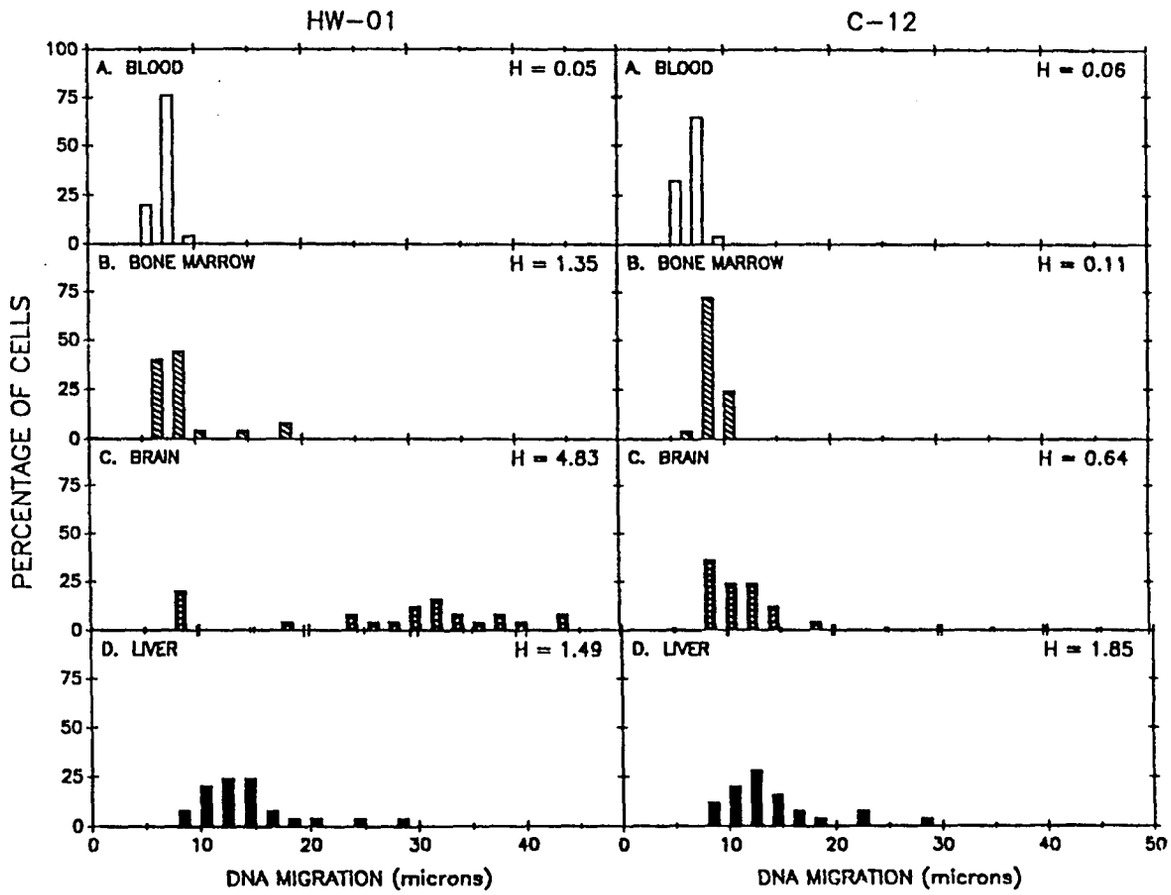

Figure 53. Distribution of DNA Migration Lengths for Blood, Bone Marrow, Brain and Liver Cells Sampled from a Representative Animal for the Hazardous Waste and Control Sites. Data based on 25 cells per tissue. The width of each bar represents 2 microns.

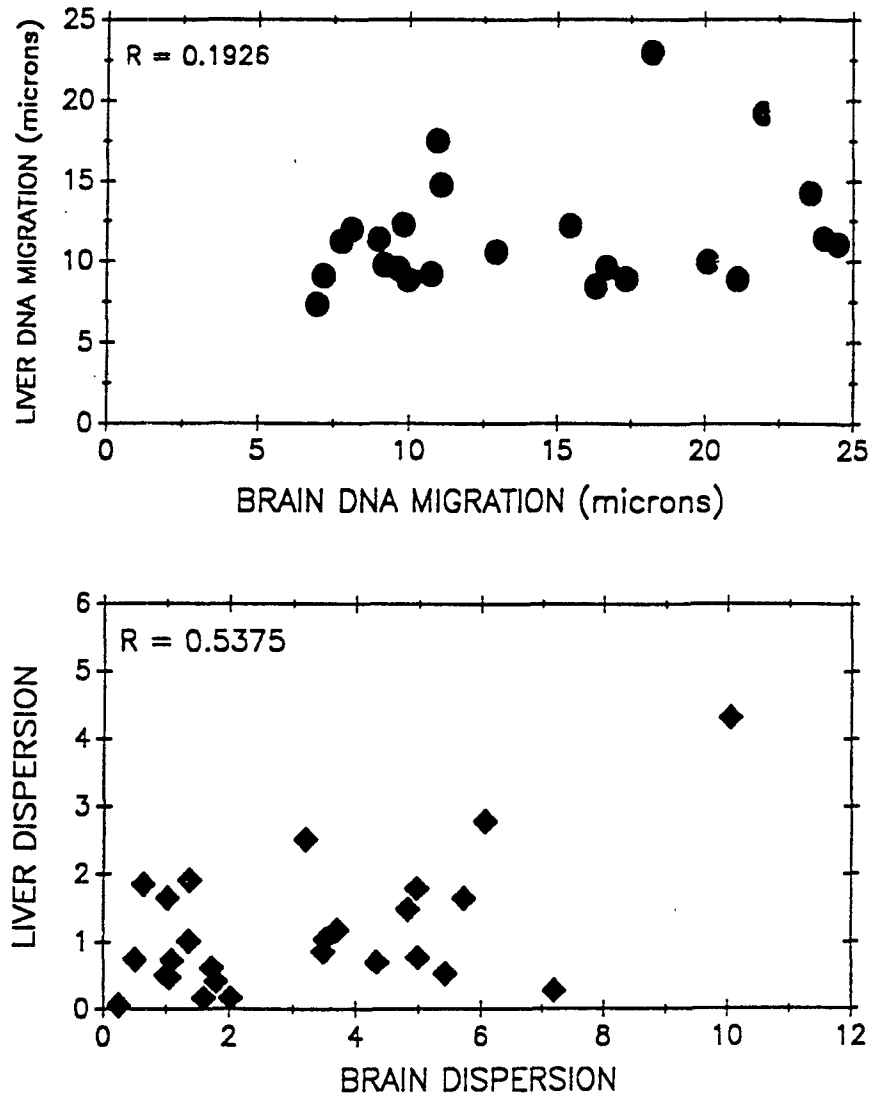

Figure 54. Correlation Between of DNA Migration Length and Dispersion Coefficient for Brain and Liver Cells in each Animal Collected at the Hazardous Waste and Control Sites. Each symbol represents the response in an individual animal.

9.0 CONCLUSION

While technical difficulties were encountered during the development and application of the SCG technique to in vitro and in vivo studies, the results presented in this technical summary indicate that many of the problems have been surmounted and that the approach should be of considerable value to scientists attempting to evaluate animal and human populations for DNA damage induced by genotoxic agents as a consequence of environmental pollution.

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Tice, R.R., Hirai, O., Andrews, P.W., and Nauman, C.H. (1990) The single cell gel (SCG) assay: a sensitive technique for human biomonitoring. Presented at the Twenty-First Annual Meeting of the Environmental Mutagen Society, Albuquerque, NM, March 25-29.

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11.0 SCG PRESENTATIONS

1. The Single Cell Gel (SCG) Assay: A Sensitive Technique for Evaluating Intercellular Differences in DNA Damage and Repair, invited platform presentation by R.R. Tice, 5th International Conference on Environmental Mutagens (July 10-15, 1989; Cleveland, OH).

2. The Single Cell Gel (SCG) Assay: A New Tool for Detecting Organ-Specific Levels of DNA Damage Induced By Genotoxic Agents, poster presentation by P.W. Andrews, R.R. Tice, and C.H. Nauman, 5th International Conference on Environmental Mutagens (July 10-15, 1989; Cleveland, OH).

3. The Single Cell Gel Assay: A Sensitive Technique For Evaluating Intercellular Differences in DNA Damage and Repair, invited platform presentation by R.R. Tice, P.W. Andrews, and N.P. Singh, Conference on Methods for the Detection of DNA Damage in Human Cells (October 1-4, 1989; Brookhaven National Laboratory, Upton, NY).

4. The Single Cell Gel Technique: A New Method for Analyzing DNA Damage and Repair, invited platform presentation by R.R. Tice, Genotoxicity and Environmental Mutagen Society Seventh Annual Meeting (October 19, 1989; Raleigh, NC).

5. Detection of Chemically-Induced DNA Damage in Individual Cells, invited seminar by R.R. Tice, R.J. Reynolds Tobacco Company (November 15, 1990; Winston-Salem, NC).

6. The Single Cell Gel Assay: A New Technique For Evaluating Intercellular Differences in DNA Damage and Repair, invited seminar by R.R. Tice (December 20, 1989; Brookhaven National Laboratory, Upton, NY).

7. The Single Cell Gel (SCG) Assay: An Electrophoretic Technique for the Detection of DNA Damage in Individual Cells, invited platform presentation by R.R. Tice, P.W. Andrews, and N.P. Singh, 4th International Conference on Biological Reactive Intermediates (January 14-17, 1990; Tucson, AZ).

8. The Single Cell Gel Assay: A New Method for the Detection of DNA Damage in Human Populations, invited seminar by R.R. Tice, University of North Carolina- US Environmental Protection Agency Joint Epidemiology Seminar Series (February 6, 1990; Chapel Hill, NC).

9. The Single Cell Gel Technique: A New Method for Evaluating Intercellular Differences in DNA Damage and Repair, invited seminar by R.R. Tice, U.S. Food and Drug Administration (March 8, 1990; Washington, DC).

10. Detection of Acrylamide-Induced Organ-Specific Levels of DNA Damage Using A New Single Cell Electrophoretic Technique, invited seminar by R.R. Tice, U.S. Environmental Protection Agency (March 9, 1990; Washington, DC).

11. The Single Cell Gel (SCG) Assay: A Sensitive Technique for Human Biomonitoring, platform presentation by R.R. Tice, O. Hirai, P.W. Andrews, and C.H. Nauman, Twenty-First Annual Meeting of the Environmental Mutagen Society (March 25-29, 1990; Albuquerque, NM).

12. In Vitro DNA Damage in Peripheral Blood Leukocytes as Measured by the Single Cell Gel (SCG) Assay, platform presentation by P.W. Andrews R.R. Tice, and C.H. Nauman, Twenty-First Annual Meeting of the Environmental Mutagen Society (March 25-29, 1990; Albuquerque, NM).

13. A New Method for Evaluating Intercellular Variability in DNA Damage, invited seminar by R.R. Tice, University of New Mexico Toxicology Program (March 30, 1990; Albuquerque, NM).

14. The Single Cell Gel (SCG) Assay: An Electrophoretic Technique for the Detection of DNA Damage in Individual Cells, invited seminar by R.R. Tice (June 26, 1990; Merck, Sharp and Dome Pharmaceutical Co., West Point, PA).

15. The Single Cell Gel (SCG) Assay: A New Method for the Detection of DNA Damage Resulting From Hazardous Pollutants, invited presentation by R.R. Tice (August 14-15, 1990; U.S Army Research Methods Branch Workshop, Frederick, MD).

16. The Single Cell Gel (SCG) Assay: A New Method for Evaluating Intercellular Differences in DNA Damage and Repair, invited seminar by R.R. Tice (August 30, 1990; Argonne National Laboratory, Chicago, IL).

17. Detecting DNA Damage in Single Cells Using a Microgel Electrophoresis Technique, invited seminar (September 7, 1990; Bristol-Myers Pharmaceutical Co., Syracuse, NY).

18. DNA Damage Evaluation Using the Rodent In Vitro Hepatocyte Culture System and the Single Cell Gel (SCG) Electrophoretic Assay. Presented at the Eight Annual Meeting of the Genotoxicity and Environmental Mutagen Society, October 25, 1990, Raleigh, NC.

19. Evaluation of DNA Damage in Golden Mice (Ochrotomys Nuttalli) Inhabiting a Hazardous Waste Site Using the Single Cell Gel (SCG) Assay. Presented at the Eight Annual Meeting of the Genotoxicity and Environmental Mutagen Society, October 25, 1990, Raleigh, NC.

20. Evaluation of Chemically-Induced DNA Damage in Germ Cells of Male Mice Using the Single Cell Gel (SCG) Assay. Presented at the Eight Annual Meeting of the Genotoxicity and Environmental Mutagen Society, October 25, 1990, Raleigh, NC.

12.0 SCG ABSTRACTS/PUBLICATIONS/MANUSCRIPTS IN PREPARATION

1. Andrews, P.W., Tice, R.R., and Nauman, C.H., (1989) The Single Cell Gel (SCG) Assay: A New Tool for Detecting Organ-Specific Levels of DNA Damage Induced By Genotoxic Agents, Environ. Molec. Mutagenesis 14, S15, 10.

2. Tice, R.R., Hirai, O., Andrews, P.W., and Nauman, C.H. (1990) The Single Cell Gel (SCG) Assay: A Sensitive Technique for Human Biomonitoring, Environ. Molec. Mutagenesis 15, S17, 60.

3. Andrews, P.W., Tice, R.R., and Nauman, C.H. (1990) In Vitro DNA Damage in Peripheral Blood Leukocytes as Measured by the Single Cell Gel (SCG) Assay, Environ. Molec. Mutagenesis 15, S17, 6.

4. Tice, R.R., Andrews, P.W., and Singh, N.P. (1990) The Single Cell Gel Assay: A Sensitive Technique For Evaluating Intercellular Differences in DNA Damage and Repair, In: Methods for the Detection of DNA Damage in Human Cells (B. Sutherland, J. Sutherland, R.B. Setlow, and A. Woodhead, eds.), Plenum Publishing Co., New York. In Press.

5. Tice, R.R., Andrews, P.W., Hirai, O., and Singh, N.P. (1990) The Single Cell Gel (SCG) Assay: An Electrophoretic Technique for the Detection of DNA Damage in Individual Cells.
In: Biological Reactive Intermediates IV (R. Snyder, ed.). In Press.

6. Tice, R.R., Andrews, P.W., Croom, D.K., and Nascimbeni, B. (In Preparation) The Single Cell Gel Technique: I. Monitoring of Organ-Specific Levels of DNA Damage Induced by Acrylamide in Mice.

7. Andrews, P.W., and Tice, R.R. (In Preparation) The Single Cell Gel Technique: II. Detection of DNA Damage Induced by Acrylamide, Trichloroethylene and Dimethylbenzanthracene in CHO cells in vitro.

8. Hirai, O., Noguchi, H., and Tice R.R. (in preparation) Application of the Single Cell Gel Electrophoresis (SCG) Technique to the Rodent In Vitro Hepatocyte Culture System: 1. Kinetics of Cyclophosphamide-Induced DNA Damage in Mouse Parenchymal Cells.

9. Hirai, O., Noguchi, H., and Tice R.R. (in preparation) Application of the Single Cell Gel Electrophoresis (SCG) Technique to the Rodent In Vitro Hepatocyte Culture System: 2. Comparison of DNA Damage Induced in Parenchymal and Nonparenchymal Cells by Direct and Indirect Acting Genotoxic Chemicals.

10. Hirai, O., Noguchi, H., and Tice R.R. (in preparation) Application of the Single Cell Gel Electrophoresis (SCG) Technique to the Rodent In Vitro Hepatocyte Culture System: 3. Comparison of DNA Damage Induced in Mouse and Rat Hepatocytes by 2-Acetylaminofluorene, 4-Acetylaminofluorene, Cyclophosphamide, Dimethylnitrosamine, Benzo(a)pyrene, Ethylmethanesulphonate, and Mitomycin C.

11. Tice, R.R., Phillips, M., Andrews, P.W., and Croom, D.K. (In Preparation) Increased Levels of DNA Damage in Free-Living Rodents Inhabiting a Hazardous Waste Site.

12. Tice, R.R, Strauss, G.S.H., , Everson, R., and Peters, W.H. (In preparation): High-dose Combination Alkylating Agents with Autologous Bone Marrow Support in Patients with Breast Cancer: Preliminary SCG Assessment of DNA Damage in Individual Peripheral Blood Lymphocytes.