PROJECT TITLE: MOLECULAR DOSIMETRY FOR CARCINOGENS

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PRINCIPAL INVESTIGATORS: SURESH P.KRISHNAN

STUDENT ADVISOR: DR. DAVID WARSHAWSKY

CO-INVESTIGATORS: DR. GLENN TALASKA,
DR. GORDON LIVINGSTON.

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LIST OF ABBREVIATIONS

BaP- Benzo(a)pyrene

CA- Carcinogen-DNA adducts

MN- Micronuclei

SCE- Sister chromatid exchange

DBC- 7H-Dibenzo(c,g)carbazole

DMBA- 7,12-Dimethylbenz(a)anthracene

7,8-BF - 7,8-Benzoflavone

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SIGNIFICANT FINDINGS

1. DBC induces a significant increase in MN over solvent controls [N=4].
2. DMBA induces a significant increase in MN over solvent controls [N=3].

   In the case of DBC and DMBA, the BNF decreases with a concomitant increase in micronucleated cells indicating that there is some selection against these cells.
3. The adduct pattern, seen in cultured mouse keratinocytes, for DBC is similar to that seen in mouse skin.
4. There is a strong correlation between carcinogen-DNA adduct levels and MN induction for DBC [r=0.6620].
5. Combined adduct 3 and adduct 6 levels correlated better with MN induction (r=0.7251) than total adduct levels. This indicated that specific adducts may be better predictors of genotoxicity than combined adduct levels.
6. There is a single DMBA adduct in mouse keratinocytes as opposed to 4 adducts seen in mouse (BALB/c ANN) skin by Randerath *et. al.*, 1985.
7. There is a stronger correlation between DMBA adduct levels and MN in mouse keratinocytes [r=0.7285].
8. Preliminary studies indicate that administration of 7,8-BF (an inhibitor of CYP 1A1 and CYP 1A2 concomitantly with DBC causes a partial inhibition of MN when compared to appropriate controls.

   This partial inhibition indicated that some of the MN may

have resulted from covalent modification of DNA. Furthermore, it is known that DBC is mutagenic in a forward mutation assay (Schoeny & Warshawsky, 1987).

9. On the other hand, preliminary studies indicate that 7,8-BF does not inhibit DMBA-induced MN formation. It is known that DMBA is a more potent inducer of chromosomal aberrations than BaP. Hence, it is quite possible that DMBA adducts may be converted more efficiently to MN than DBC adducts. Additional studies are underway to determine if the above mentioned lack of inhibition of DMBA-induced MN is reproducible.

CONCLUSION:

A: **CLASTOGENIC THEORY OF MN FORMATION:** The strong statistical association between carcinogen-DNA adducts and micronuclei for both DBC and DMBA indicates that the two end points may be causally related for both compounds. In other words, covalent modification of DNA (carcinogen-DNA adducts) may explain, at least in part, the induction of cytogenetically observable lesions (micronuclei) for DBC as well as DMBA. The reasons for the lower levels of DMBA adducts could be that DMBA-adducts are unstable and hence may be undetectable by the ^{32}P-postlabelling assay. Note: Unstable adducts were predominantly found in mouse skin following exposure to DMBA [Devanesan *et. al.*, 1993]. However, the DMBA-induced MN levels were at comparable levels to that of DBC or at some doses even higher. The reasons for this result may be that 1) DMBA has a tumorigenic potency higher than that of BaP by one order of magnitude. 2) DMBA

is known from the literature to be a more potent inducer of chromosomal aberrations than BaP (Nishi *et.al.*, 1980).

B. **ANEUPLOIDOGENIC THEORY OF MN FORMATION:** An inhibitor of cytochrome P-450 [7,8-BF] causes a partial inhibition of DBC-induced MN. This result indicates that some of the MN may have arisen by a mechanism independent of DNA adduct formation. It is known from the literature that MN may also arise as a result of loss of entire chromosomes due to damage to the spindle apparatus (Eastmond & Tucker, 1989). This may explain the excess MN seen which could not be inhibited by 7,8-BF. In addition, preliminary data indicates that DMBA-induced MN could not be inhibited by 7,8-BF at all. Hence, DMBA could be largely aneuploidogenic.

A dose response study with various concentrations of inhibitor would enable us to more accurately assess the relative contributions made by these two mechanisms to MN induction.

USEFULNESS OF FINDINGS

The similarities in the adduct patterns seen in this study with published results in mouse skin indicate a similar method of bio-activation for both compounds. This lends further credence to the popular belief that in-vitro systems could be used to predict in-vivo genotoxicity. In contrast to MN, DNA adducts have been used as a biomarker in human population bio-monitoring. However, this study was the first to validate the usefulness of the MN assay as a marker of biologically effective dose in an easily accessible target organ. The scatter plot of individual data points indicate a linear relationship between the two end points. This is in sharp contrast to the "threshold theory" proposed in the literature. In other words, it has been suggested that there is a threshold adduct dose above which the two assays complement each other (Arce et.al., 1987; Wiencke et. al., 1990). The data generated in this study does not seem to suggest such a threshold phenomenon. However, this does not mean that such a situation does not exist in vivo. The results obtained in this study provide further impetus to examining more closely the relationship between the two biological markers of exposure (adducts and MN) in primary cultures of human keratinocytes obtained through non-invasive skin biopsies from workers exposed to carcinogen-DNA adducts in an occupational setting.

ABSTRACT

The objective of this study was to examine the relationship between DNA adducts and MN in primary cultures of mouse keratinocytes treated separately with BaP, DBC and DMBA. The specific aims were:

1. **A) DOSE RESPONSE STUDY:** to determine of the ability of BaP, DBC and DMBA separately to induce MN in cultured mouse keratinocytes.

   B) TIME COURSE STUDY: to determine of the time for maximal MN expression.

2. to determine the major adducts formed by BaP, DBC and DMBA in mouse keratinocytes [^{32}P-postlabelling].
3. to establish dose-response curves separately for carcinogen-DNA adducts and MN for BaP, DBC and DMBA.
4. to perform statistical analyses to determine the strength of the association between the two end points.
5. to elucidate the mechanism of MN formation by using an inhibitor of cytochrome P-450 [7,8 Benzoflavone].

The results indicate that DBC and DMBA induce MN significantly over controls. DBC [24 hours] and DMBA [6 hours] have different optimum times for MN formation. There is a strong statistical association between CA and MN for DBC (r=0.6620). Furthermore, combined adduct 3 and adduct 6 levels are better predictors of genotoxicity than total adduct levels (r=0.7251). In addition, there is a strong correlation between CA and MN for DMBA (r=0.7285). There

is a partial inhibition of DBC-induced MN by 7,8-BF, an inhibitor of cytochrome P-450. Taken together, the data suggests that the covalent modification of DNA (carcinogen-DNA adducts) could account for at least some of the cytogenetically observable lesions [MN induction].

BODY OF REPORT WITH CONCLUSIONS

Dermal absorption may be a significant route of exposure for many carcinogens in the occupational setting. [In addition, the onset of disease (e.g., cancer) may be the result of a combination of genetic and environmental factors (deficiency of DNA repair enzymes in Xeroderma pigmentosum (XP) patients and increased susceptibility to UV light-induced skin cancer)]. For example, occupations such as coal gasification, coke production, and exposure to complex mixtures of chemicals (coal tar, coal tar pitches, certain mineral oils, shale oils and soots may increase the risk of skin cancer [Montesano et. al., 1987]. On the other hand, measurement of airborne levels of pollutants may not accurately predict dermal absorption of xenobiotics in the workplace. Hence, it is necessary to develop and validate a biological marker of exposure which can integrate individual differences in absorption, distribution, metabolism and excretion. One such biological marker of exposure is the measurement of carcinogen-DNA adduct [CA] levels.

Carcinogen-DNA adducts are formed as a result of metabolism of chemical carcinogens, by cytochrome P-450 enzymes, and the binding of the resultant electrophilic intermediates to DNA. The net CA levels depend on the extents of metabolic activation, detoxification and DNA repair. On the other hand, it has been found that the CA levels may be similar in both target and non-target organs [Jeffrey et. al., 1990]. Furthermore, it is believed that initiation/mutation is the result of persistent DNA lesions not being repaired prior to the onset of DNA replication. In addition,

mutagenic lesions may be introduced in the DNA through error-prone repair [Gill et. al., 1991]. Finally, intra-genic mutations and genomic mutations may also be important in carcinogenesis [Heddle et. al., 1983].

Hence, it is imperative that biological markers of exposure be related to markers of early effect following exposure. One such marker of early effect is the induction of micronuclei [MN]. MN are round to oval bodies found within the cytoplasm but outside the main nucleus. The resemble the main nucleus in shape, texture, and staining properties and may be detected as markers of mis-segregated chromatin. MN may be formed either when acentric fragments from broken chromosomes or when entire chromosomes get excluded from the main nucleus at the time of cell division (Eastmond & Tucker, 1989). Finally, MN have been shown to be induced following exposure to genotoxic chemicals (He & Baker, 1991).

Several studies have been performed that relate CA levels to cytogenetic end points (sister chromatid exchanges and MN) [Van der Poll et. al., 1989; Wiencke et. al., 1990] and to genotoxic end points (mutations) [Parks et. al.,1986] in several target and non-target organs. However, This study was the first to determine the possible relationship between CA and MN in *mouse skin*. Mouse skin is considered to be an excellent model for understanding the mechanisms of epithelial carcinogenesis. It is known that the majority of cancers arise from surface epithelia. Mouse keratinocytes are the predominant cell type in the epithelial layer

of mouse skin. However, there is a great deal of functional heterogeneity in this cell layer. The basal cells have the most proliferative potential and hence conditions were established and optimized for obtaining a monolayer of murine keratinocytes in culture [Figures 2 & 3]. The expression of MN formation requires that the assay be performed on cells that have divided once. Hence, conditions were optimized for obtaining maximal numbers of binucleated cells. It was found that the cells have to be cultured, for at least 72 hours, for obtaining maximal numbers of binucleated cells in acetone treated controls [Figure 4].

Skin painting of various doses of benzo(a)pyrene (BaP) and determination of MN induction revealed that BaP failed to induce MN significantly over acetone controls [Figure 5] It was then decided to determine the ability of BaP to induce MN following *in-vitro* incubation for 24 hours.

Figure 6 is a schematic diagram for the DNA adduct and the MN assay. Mouse keratinocytes are isolated and the medium is changed 24 hours later. The medium is replaced either with medium containing 0.5% DMSO or with medium containing varying doses of BaP,7H-dibenzo(c,g)carbazole(DBC) and 7,12-dimethylbenz(a) anthracene (DMBA). The cells,for DNA adduct analysis, are trypsinized and kept frozen at -80°C for subsequent DNA isolation and determination of DNA adduct levels by the ^{32}P-post labeling assay. The cells for the MN assay are cultured for another 72 hours before being fixed in 100% methanol and stained with acridine orange [Figure 6]. Acridine orange is a differential stain with the

cytoplasm staining orange and the nucleus staining green.

Low doses of BaP were negative in inducing MN over controls [Figures 7 & 8]. Higher non-cytotoxic doses of BaP were also negative in inducing MN over DMSO controls [Figure 9]. The lack of induction of MN over controls may not have been due to the inability of BaP to be metabolized by murine keratinocytes. This is demonstrated by the fact that anti-BPDE is, at best, an inefficient inducer of MN over controls [Figure 10]. However, primary cultures of murine keratinocytes have the ability to express MN formation. This was demonstrated by the fact that adriamycin (a DNA strand breaker- a direct acting compound which does not require metabolic activation) produces a significant increase in MN over controls [Figure 11]. Furthermore,exposure of mouse keratinocytes to DBC for 24 hours [0-2500 ng/ml] produces a dose-dependent increase in MN over controls [Figure 12]. In addition, time course experiments show that a single dose of DBC produces a statistically significant increase in MN over controls [Figure 13]. In addition, exposure to DMBA for 6 hours (0-256 ng/ml) produces a statistically significant increase in MN over controls [Figure 14]. Furthermore, time course experiments also show that there is a statistically significant increase in MN at all times tested [Figure 15]. In addition, exposure to several doses of DMBA [0-256 ng/ml] for 24 hours produces a dose-dependent increase in MN over solvent controls [Figure 16]. At the highest [256 ng/ml] dose tested, DMBA produces an approximately two fold higher level of MN when compared to DBC.

Experiments were designed to test the hypothesis that the two

end points were related. Experiments were first performed to establish the fact that the growth characteristics of the cells were the same on dishes as well as on slides [Figure 17]. The dose response curves and the scatter plot for DBC-induced adducts and MN indicate that the two end points are correlated [Figures 18 & 19] with a Spearman rank correlation coefficient of 0.6. The individual data points for the different DBC adducts vs MN yielded the following correlation coefficients:

1. Adduct 2	0.3488	[Figure 20]
2. Adduct 3	0.5807	[Figure 21]
3. Adduct 6	0.5788	[Figure 22]
4. Adduct 3 + Adduct 6	0.7251	[Figure 23]
5. Adduct 2 + Adduct 6	0.4819	[Figure 24]
6. Adduct 2 + Adduct 3	0.4363	[Figure 25]
7. Adduct 2 + Adduct 3 + Adduct 6	0.5512	[Figure 26]

The dose response curves as well as the scatter plots for DMBA indicate that the two end points are correlated [Figure 27 & 28] with a Pearson's correlation coefficient of 0.7285. In addition, there is a good dose response relationship for DMBA-induced MN while there is a poor relationship for carcinogen-DNA adducts (Figure 29). However, when 6 outliers were deleted, the correlation between DMBA adducts and MN improves significantly and is 0.6810 (Figure 30).

CONCLUSIONS: CA and MN are strongly correlated for DBC (r=0.59) and DMBA (r=0.73). There is a partial inhibition of DBC-induced MN by

an inhibitor of cytochrome P-450 [Table 2]. This indicates that MN formation could, at least in part be explained due to covalent modification of DNA.

A dose-response study with various concentrations of inhibitor would enable us to more thoroughly evaluate the relative contributions made by the clastogenic and/or the aneuploidogenic components of DBC and DMBA.

METHODS

ISOLATION OF PRIMARY CULTURES OF MURINE KERATINOCYTES:

Mouse keratinocytes were isolated from female HSD:ICR(Br) [Harlan Labs] mice [6-7 weeks old]. The cells were pooled from 6 mice per experiment. The mice were sacrificed with an intraperitoneal injection of sodium pentobarbital [5 mg/ml/mouse]. The backs of mice were shaved with electric clippers. The mice were then dipped in Betadine solution [Hospital Supplies] for 2 minutes and rinsed several times with millipore water. This step was repeated. The final two rinses were in 70% ethanol [2 minutes each]. In a laminar flow hood, the skins were aseptically excised, using autoclaved surgical instruments. The skins were transferred to a specimen cup containing Ca^{++} Mg^{++} free phosphate buffered saline (PBS) which had 2X gentamicin [antibiotic] in it. The skins, one at a time, were transferred to a 100 mm petri dish and the fat was scraped off from the dermal side (using a scalpel with a #22 blade) until the skin appeared translucent. The skins were then cut into square pieces (1 cm across) and floated dermis side down in 0.25% trypsin solution (Gibco) at 31°C for 3 hours. After 3 hours, the epidermis was scraped from the dermis into medium containing 10% fetal bovine serum (to neutralize the activity of trypsin). The scraping was done in a square petri dish, tilted at an angle of 10-20° using a scalpel with a new #22 blade. The scraping takes about 2 hours. The scraped epidermis, in 30 ml of medium, were stirred (100 rpm) for about 30 minutes in a cup which has a magnetic stirrer in it. The

cell suspension was then filtered through a 70 μM nylon mesh into a 50 ml centrifuge tube. The cell suspension was then centrifuged at 1600 rpm for 10 minutes. The resultant cell pellet was taken up in 20 ml of medium and triturated 15-20 times. One ml of this cell suspension was transferred to another centrifuge tube and 9 ml of medium was added. This cell suspension was in turn triturated 15-20 times, an aliquot was taken for determining cell count and viability (trypan blue exclusion) and approximately 3 x 10^6 cells were plated on collagen [Recipe: 90 ml medium; 1 ml vitrogen; 1ml fibronectin; 10 ml bovine serum albumin (1 mg/ml solution); 1 ml of 2M HEPES] coated slides. **NOTE:** Immunocytochemical staining, using standard procedures, of mouse keratinocytes re-confirmed the fact that the cells were predominantly keratinocytes (data not shown).

The time course experiments were performed as follows: The medium was replaced, 24 hours later, with medium with or without the carcinogen dissolved in 0.5% DMSO. The solvent controls had medium with 0.5% DMSO in it. The doses used for the experiments were as follows :

BaP: 100; 150 and 750 ng/plate. DBC: 15; 150; and 1500 ng/plate. DMBA: 48; 96; 192; 384 ng/plate. The cultures were dosed in yellow light to prevent photo-oxidation of the carcinogen.

DETERMINATION OF MICRONUCLEI LEVELS:

After carcinogen exposure (6 or 12 or 24 hours), the medium was replaced with medium containing cytochalasin-B (to obtain binucleated cells) for 72 hours. **NOTE:** In all the cases, the duration of exposure of the cells to cytochalasin-B was kept

constant. After 72 hours, the slides were washed in PBS and fixed in 100% methanol for 2 minutes. The slides were then stained with acridine orange (Sigma) for 1 minute, rinsed with PBS and mounted in PBS. The edges of the cover slip were sealed with rubber cement. MN scored were normalized to the number of MN in 1000 binucleated cells. **NOTE:** The dose response experiments, for combined adduct and MN measurements, were performed after carcinogen exposure for 24 hours (BaP/DBC/DMBA) and for 6 hours (DMBA). The cells, for the DNA adduct analysis, were trypsinized and kept frozen at -80°C for subsequent DNA isolation and ^{32}P-postlabelling. The cells for the determination of MN levels were treated and analyzed as for the time course experiments.

^{32}P-POSTLABELLING

ISOLATION OF DNA:

DNA was isolated following the phenol extraction procedure of Gupta *et. al.*, 1984 with some modifications. The cells, kept frozen at -80°C in 15 ml centrifuge tubes, were thawed out and 1.5 ml of 1% SDS-EDTA was added. The cells were triturated a few times using a Pasteur pipette to disaggregate the cells. The cells were transferred to numbered Corex tubes and 24 μl of 1M Tris buffer [pH 7.4] was added. The cells were homogenized using a Brinkman homogenizer [Position 4] for 10 seconds. The samples were stored in ice during this procedure. The homogenizer was rinsed thoroughly between samples to avoid cross-contamination. This procedure was followed by the addition of 30 μl of RNase A [10 mg/ml] and 10 μl of RNase T_1 [5 u/μl]. The samples were vortexed and incubated at

37°C for 1 hour. After 1 hour, the samples were taken out of the incubator and 60 μl of Proteinase K [10 mg/ml] was added. The samples were again vortexed and returned to the 37°C incubator for 1 hour. After 1 hour, the samples were returned to ice and 1.2 ml of cold phenol [saturated with 20 mM TRIS] was added to each sample. The samples were vortexed for 10 seconds and then spun, in a 4°C centrifuge [Sorvall 5B Refrigerated Superspeed Centrifuge with a SS34 rotor], at 7000 rpm for 10 minutes. The top phase, from each sample, is transferred to a correspondingly numbered Corex tube. It was critical to make sure that the thick interphase was not taken with the top phase. Phenol and Sevag (1.2 ml) in the ratio of 1:1 was added to each sample. The samples were then vortexed and spun at 7000 rpm for 10 minutes. The top layer was then transferred to a fresh Corex tube and 1.2 ml of Sevag was added. The samples were vortexed, spun and the top was transferred to another Corex tube. The samples were placed on ice and 100 μl of 4 M LiCl and 10 μl of glycogen [30 μg/μl] was added and vortexed. This procedure was followed by the addition of 1 volume of cold ethanol. The tubes were mixed with a shaking motion and then incubated at -80°C for 15 minutes. The samples were spun at 7000 rpm for 10 minutes. The 100 % ethanol was poured off and rinsed twice with cold 70% ethanol. The tubes were inverted and placed on absorbent paper and dried. The DNA pellet was then dissolved in 20-30 μl of 1/100 SSC-1 mM EDTA solution.

HYDROLYSIS:

NOTE: All the samples were labelled *"carrier-free"*[see explanation

below] with the addition of apyrase. This variant of the ^{32}P post-labelling assay does not require that the concentration of DNA be known prior to labelling.

The microfuge tubes [1.5 ml] were numbered [1A, 1B .....8A, 8B] so that each sample was done in duplicate. The enzyme digestion cocktail was prepared for 18 samples [two more than what was needed to make up for loss of enzymes due to pipetting errors] as indicated below:

2 μl enzyme mix containing 0.25 MN unit and		
2.5 μg SPD	x 18 samples	= 36 μl
2 μl 5X salts, pH = 6.0	x 18 samples	= 36 μl
2 μl H_2O	x 18 samples	= 36 μl

The components were mixed in a 1.5 ml microfuge tube, spun down and placed on ice.

The hydrolysis cocktail [6 μl] was transferred to each numbered 1.5 ml tube. All the tubes were then placed on ice. Then, the vials containing the DNA samples, stored at -80°C, were thawed and transferred to the micro-centrifuge. The DNA samples were spun down and 4 μl of the appropriate DNA sample was transferred to each tube. [NOTE: The DNA samples were vortexed between taking aliquots for the hydrolysis. This procedure served to decrease the variability in the results between duplicate samples]. The numbered tubes, containing the hydrolysis cocktail and the 4 μl of DNA were vortexed, centrifuged and placed on ice. The samples were incubated at 37°C for 6 hours. After 6 hours, the samples were stored at -80°C.

CARRIER FREE LABELLING:

In this variant of the ^{32}P-post labelling assay, the ^{32}P-ATP was limiting and the nucleotides were in vast excess. Under these conditions, the nucleotides were labelled based on the affinity of polynucleotide kinase [PNK] for the substrate. Surprisingly, the adducted nucleotides were labelled in preference to the normal nucleotides. A modification of the carrier-free labelling assay was adopted by the addition of apyrase. This enzyme aided in the complete transfer of radiolabelled phosphate in the ATP to the nucleotides. [NOTE: Under my experimental conditions, it was observed that failure to add apyrase to the labelling cocktail resulted in-complete transfer of radioactivity to the nucleotides].

LABELING COCKTAIL:

^{32}P-ATP[1]	x 18 samples	= [1]
0.28 μl PNK	x 18 samples	= 5.04 μl
1.5 μl PNK buffer	x 18 samples	= 27.0 μl
10 mM Bicine buffer[2]	x 18 samples	= [2]
TOTAL 8 μl/sample	x 18 samples	= 144 μl

[NOTE: 1. The volume of ^{32}P-ATP to be added depended on the specific activity of radiolabelled ATP on that day. 200 μCi/activity of ^{32}P-ATP = Volume of ATP to be added/sample.

2. Bicine buffer was added to make up the volume to 8 μl/sample]. All the components of the labeling cocktail, except the ^{32}P-ATP were

added to a 1.5 ml microfuge vial and placed on ice. The isotope, stored at -20°C, was taken out and left to thaw at the workstation. The required volume of radiolabel was then transferred to the labelling cocktail vial, vortexed and spun down. The hydrolyzed, DNA samples were thawed out and placed in the plexiglass, microfuge holder. The labeling cocktail [8 μl/sample] was added to each DNA sample, vortexed and spun down. The samples were then transferred to a glass microfuge storage container and incubated at 37°C for 40 minutes [PRECAUTIONS: All activities at the workstation were performed with suitable personal protective equipment i.e. gloves, lab coat and arm sleeves. The vials, at the workstation, were manipulated with long forceps. The automatic pipettors were shielded with plexiglass. Personal monitoring was performed through badges on the lab coat and ring badges. The Geiger-Mueller counter was used to check for contamination throughout the labelling]. During the incubation period, the Polyethylene Imine [PEI] plates were prepared for the normal nucleotide analysis [two plates-5 x 20 cm each; one 10 x 20 cm plate, please see below], adducted nucleotide analysis [D1] [10 x 20 cm]. Wicks [20 x 30 cm long and 2.5 cm x 20 cm] were attached to D1 and the two 5 x 20 cm plates respectively. In addition, the vials for the normal nucleotide analysis, were numbered and 746 μl of 10 mM Bicine buffer was added. These vials were then stored at 4°C. After the 40 minute incubation, the samples were taken out of the 37°C incubator and then 6 μl [17.3 u/ul] of apyrase was added to each sample. The samples were returned to the incubator for another 40 minutes.

After 40 minutes, the samples were removed from the 37°C incubator and transferred to the plexiglass microfuge holder [back row]. Then, 4 μl of each sample was transferred to the vial containing 746 μl of Bicine buffer, vortexed and spun down. These samples were left in the centrifuge till the D1 spotting was completed.

The D1 spotting takes approximately 80 minutes. In this procedure, 18 μl of each sample was withdrawn and spotted over the origin, [origins, 1.1 cm apart; 1 cm from the edge and 1.5 cm from the bottom, for the 16 samples were marked during the 40 minute incubation; fluorescent paint was used to mark the plates for subsequent identification and alignment]. The spotting was done as slowly as possible. The drop was allowed to be formed at the tip of the pipette and it was placed on the PEI plate. Sufficient time was allowed for the solution to spread out and care was taken to see that each drop was placed exactly over the origin. After each sample had been spotted, the spots were dried using a dryer. Once the spotting for all the samples were completed, the D1 plate was hung inside a chamber in the hood and allowed to develop overnight in 1.1 M LiCl [D1 solvent]. [NOTE: Care was taken to see that the D1 development times were kept as close to 14 hours as possible]. The samples, for the normal nucleotide analysis, were removed from the micro-centrifuge and placed in the microfuge vial holder. Then 5 μl/sample was applied to each sheet [3 sheets] at the appropriate origin [origins marked 1.2 cm apart; fluorescent paint used for marking the plates for subsequent identification and alignment]. After all the samples had been spotted, the sheets were developed

briefly in $^{d}H_2O$ and one sheet [10 x 20 cm] was developed in 0.15 M NaH_2PO_4 while the other two sheets [5 x 20 cm each with 2.5 x 20 cm wicks attached] were developed in 0.25 M LiCl. After development, the plates were dried , the wicks were cut and the plates were returned to the plexiglass plate holder.

After overnight development, the D1 chromatograms were removed from the solvent and the wick was cut and discarded carefully in the High Level Waste container. This procedure was performed behind plexiglass shielding and the chromatogram was handled using forceps. It should be remembered that wick retains 99% of the radioactivity. The D1 chromatograms were <u>dipped very briefly</u> in water and then dried. [NOTE: Prolonged rinses in water were causing the plates to flake off and hence were avoided]. The plates for the normal nucleotide analysis as well as the D1 plates were exposed to X-ray film in cassettes with intensifying screens. The films were exposed for 10 minutes. The plate developed in sodium phosphate indicated the extent of hydrolysis by MN-SPD [test of the efficacy of these hydrolytic enzymes]; the extent of labelling of the nucleotides [test of the efficacy of PNK and PNK buffer and apyrase] and the presence/absence of DNA in each sample. The plates developed in LiCl will be used to quantify the normal nucleotides in each sample [see below]. The D1 solvent permitted <u>only</u> the migration of normal nucleotides and the adducted nucleotides were retained at the origin. The D1 chromatogram was marked (1.75 cm from the origin) using the autoradiogram as the template. Each spot was then cut into rectangular pieces and transferred to 10 x 10 cm^2

PEI sheets. These sheets had their origins [1.5 cm from the bottom and 1.5 cm from the edge] and identification marked. The cut out was placed such that the two origins aligned and the D_1 was from left to right. The cut outs were attached to new sheets with magnets and hence this procedure is referred to as the magnet-mediated transfer technique. The D_3 chromatograms were developed briefly in water and then developed, four plates/tank, in D_3 solvent [D_3 = 85% (3.6 M Lithium Formate, 8.5 M Urea) + 15% ^dH_2O]. The plates were developed to the top of the chromatograms. After development, the plates were placed in baking trays with 1/2" de-ionized water and washed twice [7 minutes/wash]. After the two washes, the plates were dried. The D_3 solvent was poured out and the tanks were rinsed prior to the addition of D_4 solvent [85% 0.8 M LiCl; 0.5 M TRIS-HCl; 8.5 M Urea] and 15% ^dH_2O. The plates were pre-developed in H_2O and the chromatograms were developed in the D_4 solvent in a direction which was at right angles to the D_3 front. The chromatograms were developed to the top, washed twice in ^dH_2O [7 minutes/wash] and dried. The D_4 solvent was poured off, the tanks were rinsed in ^dH_2O and replaced with D_5 solvent [1.7 M NaH_2PO_4, pH 6.0]. A 2.5 cm Whatman #1 wick was attached to the top of each plate [D_5]. The D_5 plates were developed briefly in ^dH_2O and developed subsequently in the D_5 solvent [same direction as the D_4 solvent front]. The plates were dried, the wicks trimmed off, and transported to the dark room where they [4 plates/cassette] were placed in cassettes with intensifying screens and exposed to X-ray film. [Note: Before, the plates were put in cassettes, the activity on the plates was

checked using a GM counter. This was done to estimate the exposure times for autoradiography. In addition, care was taken to see that the marks for identification and alignment were in place]. The cassettes [4 cassetes/16 samples] were returned to -80°C freezer since the films were more sensitive to radio-activity at lower temperatures. The films were usually exposed for 2-3 days.

After 2-3 days, the films were removed and processed [X-ray processor]. The spots corresponding to the adducts were circled and numbered [on the film] and corresponding spots in the chromatograms were circled and cut. These cut-outs were transferred into a correspondingly numbered scintillation vial containing 5 ml of 70% ethanol. In addition, the spots corresponding to the normal nucleotides were numbered and circled on film and the corresponding spots were cut out from the chromatograms. These cut-outs were also transferred to scintillation vials. The vials were taken to the scintillation counter and counted using a computer program which had already been set up for counting ^{32}P-radionuclides. The relative adduct labelling [RAL] was determined using the following formula:

$$\langle RAL \rangle = \frac{\text{cpm in adducted nucleotides}}{\text{cpm in normal nucleotides} \times 750} \times 10^7$$

FIGURE 1

SPECIFIC AIMS

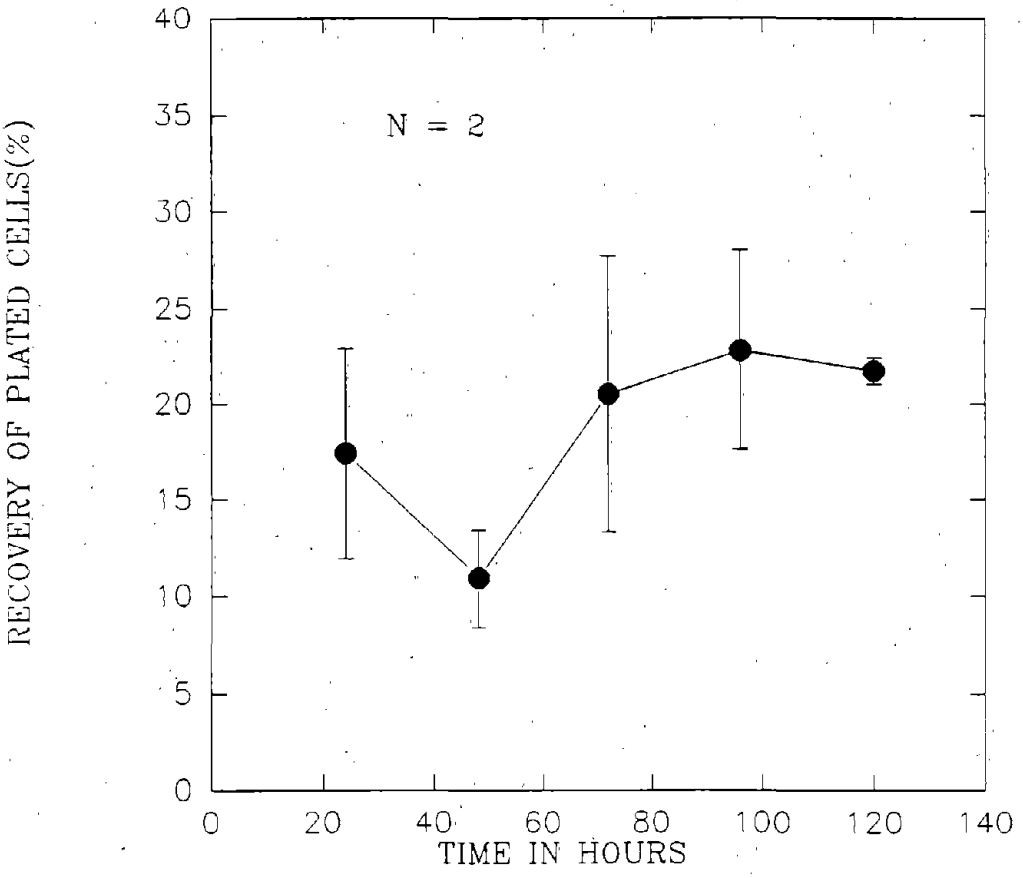
1. Determine the ability of BaP, DBC, and DMBA to cause chromosome damage in mouse keratinocytes as measured by micronuclei frequencies.

2. Establish dose-response relationships between carcinogen-DNA adducts and micronuclei for BaP, DBC and DMBA.

3. Evaluate the role of carcinogen-DNA adducts in the formation of micronuclei.

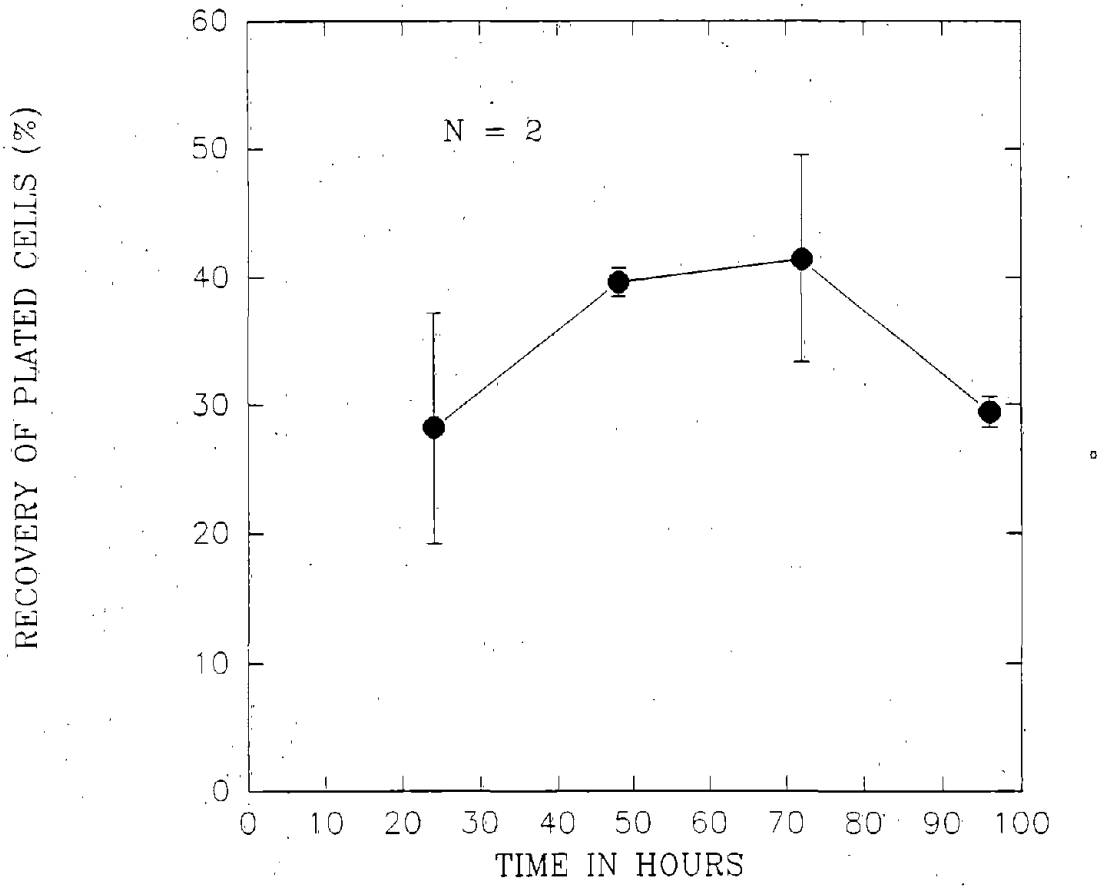
RECOVERY OF PLATED MOUSE KERATINOCYTES
FROM UNTREATED ANIMALS

FIGURE 2



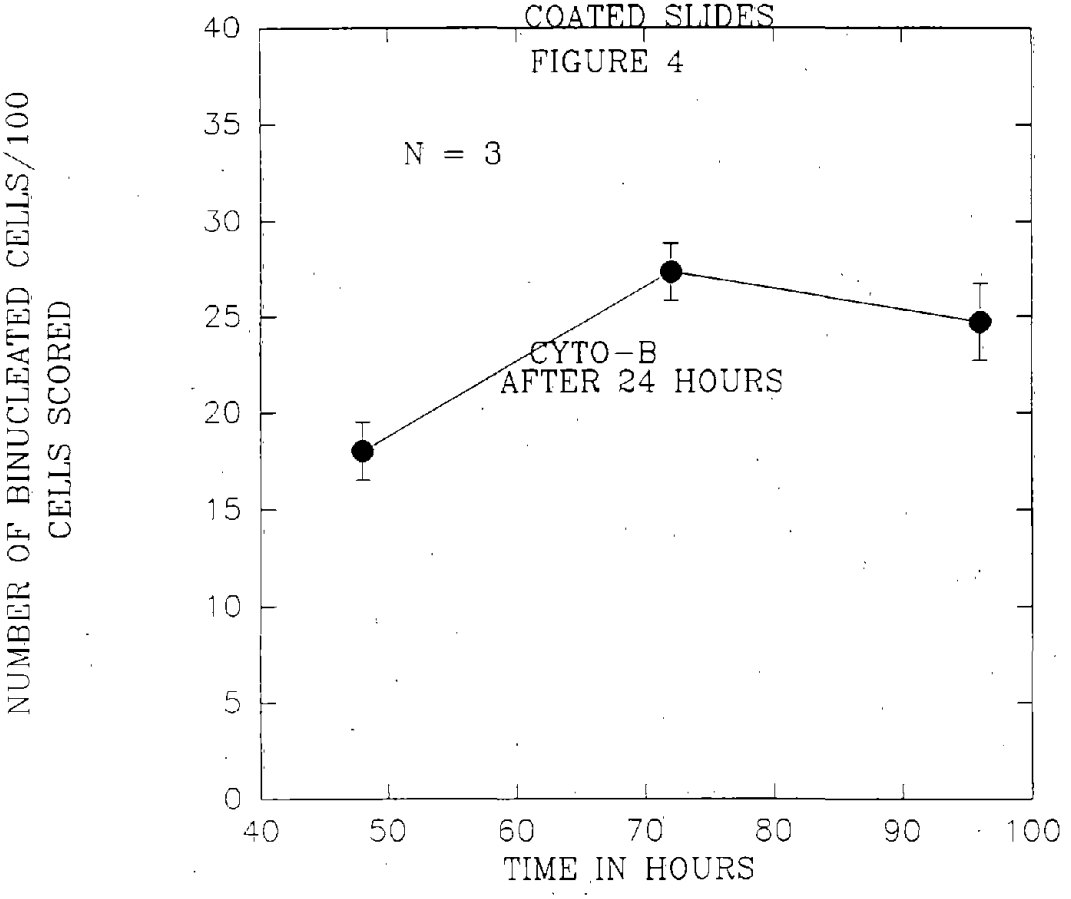
RECOVERY OF PLATED, MOUSE KERATINOCYTES FROM ACETONE TREATED ANIMALS.

FIGURE 3



OPTIMIZATION OF BINUCLEATED CELLS FOR THE MICRONUCLEUS ASSAY FOLLOWING SKIN PAINTING OF ACETONE AND PLATING OF KERATINOCYTES ON COATED SLIDES

FIGURE 4



BINUCLEATE FRACTION AND MN INDUCTION
BY BENZO[A]PYRENE FOLLOWING SKIN PAINTING
[24 HOUR EXPOSURE]
FIGURE 5

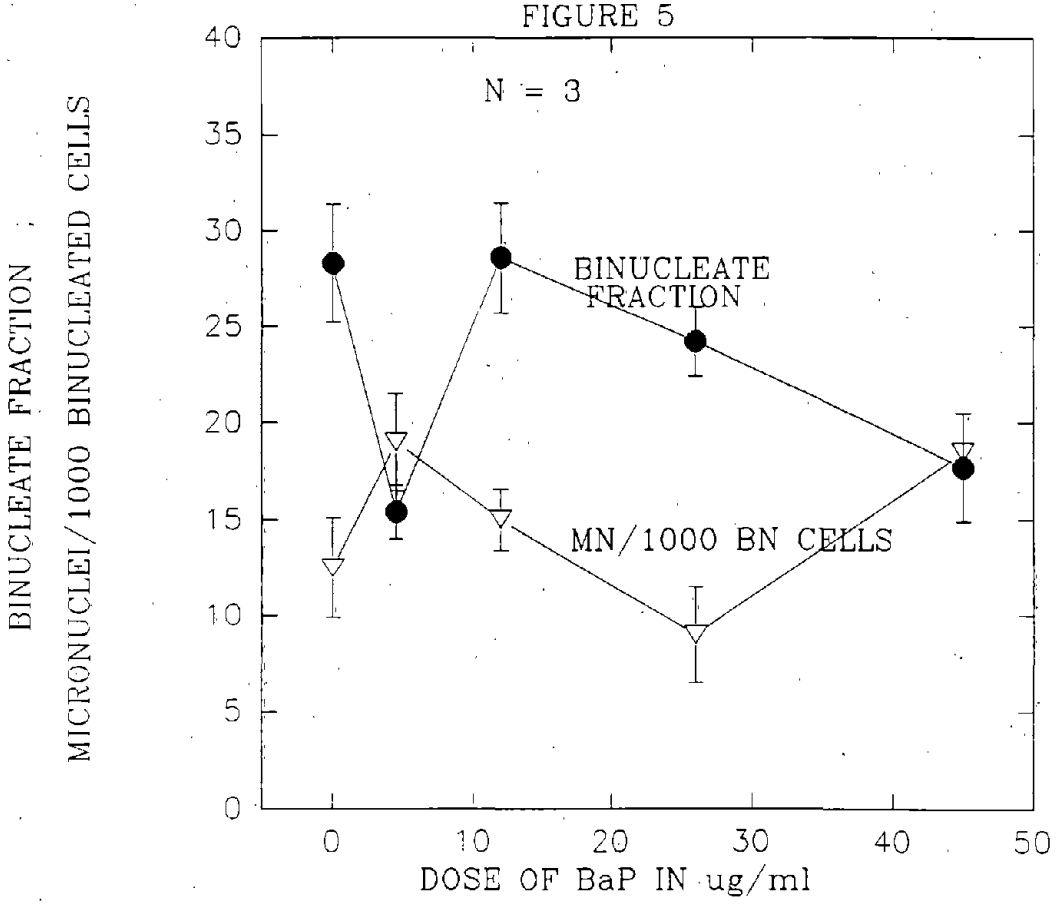
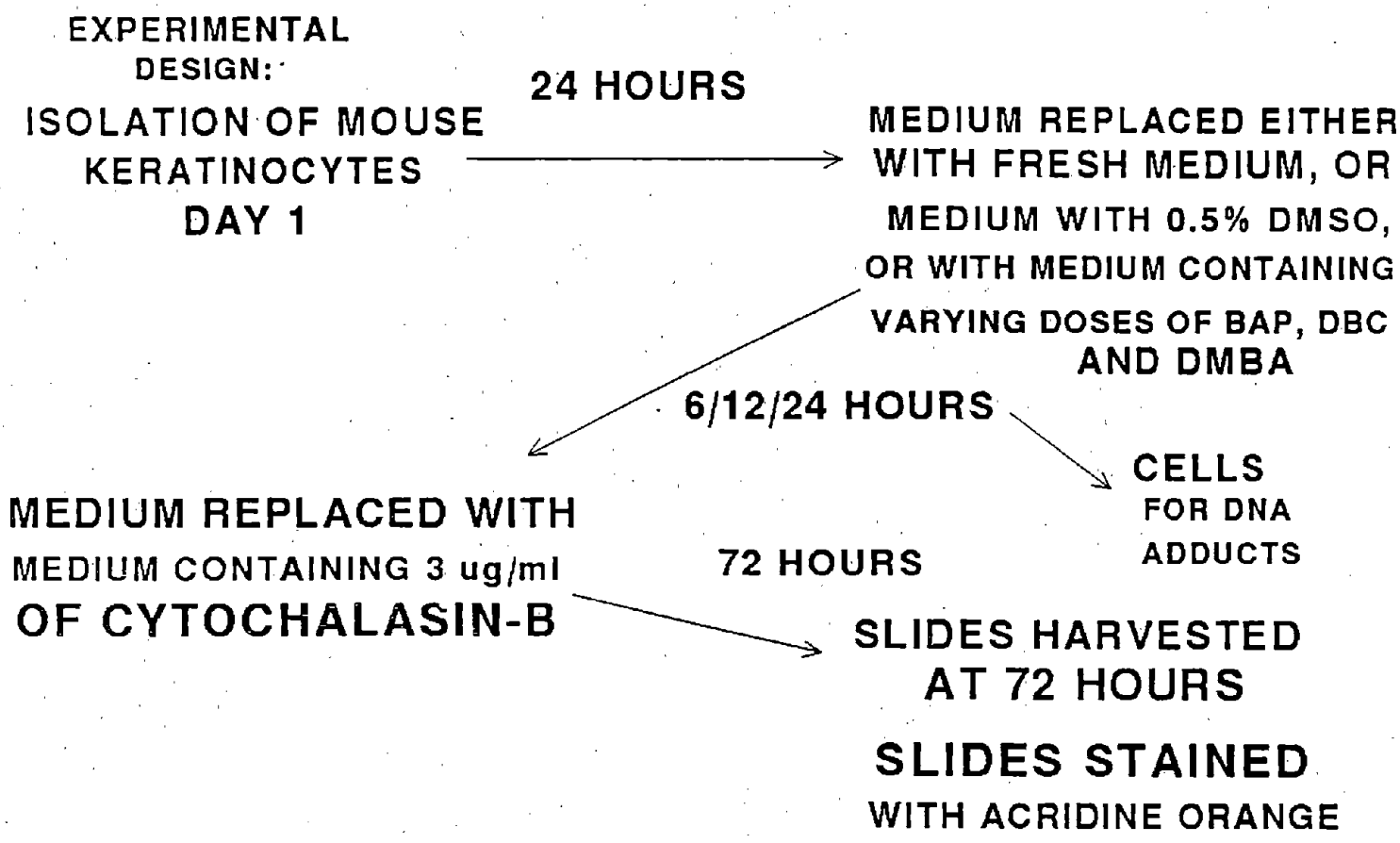


FIGURE 6

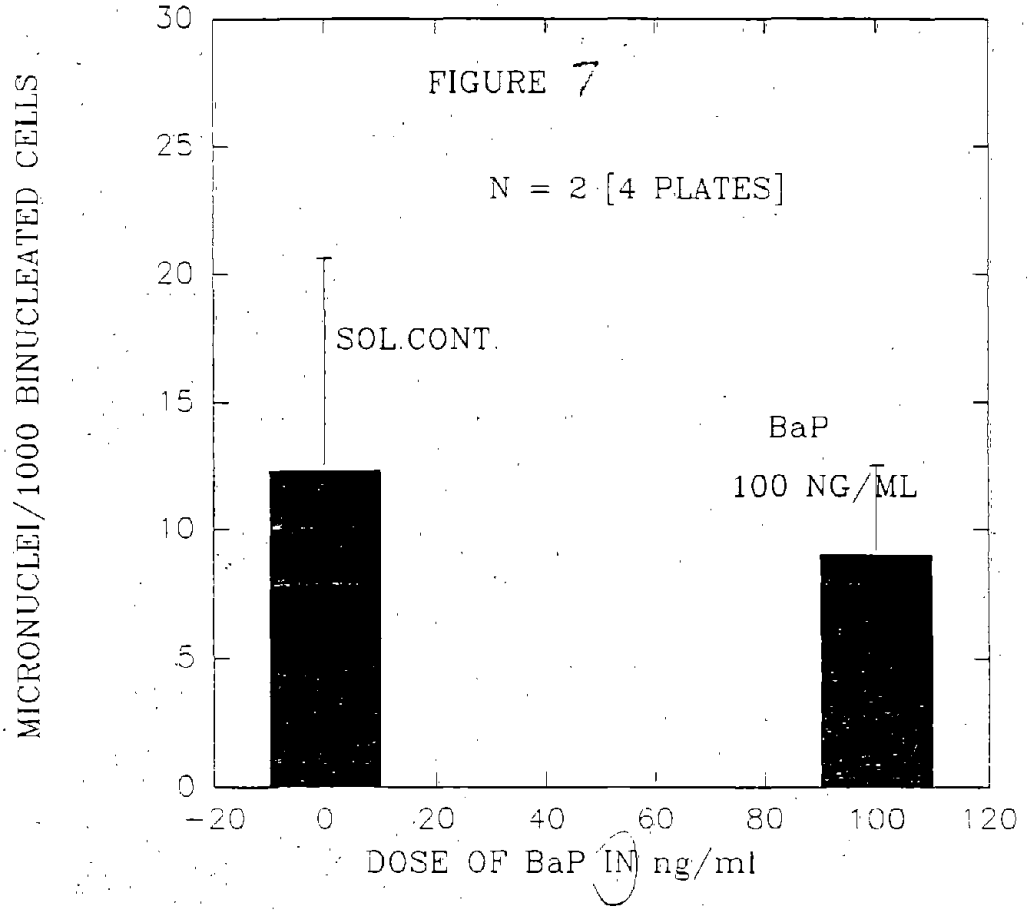
IN-VITRO EXPERIMENTAL DESIGN

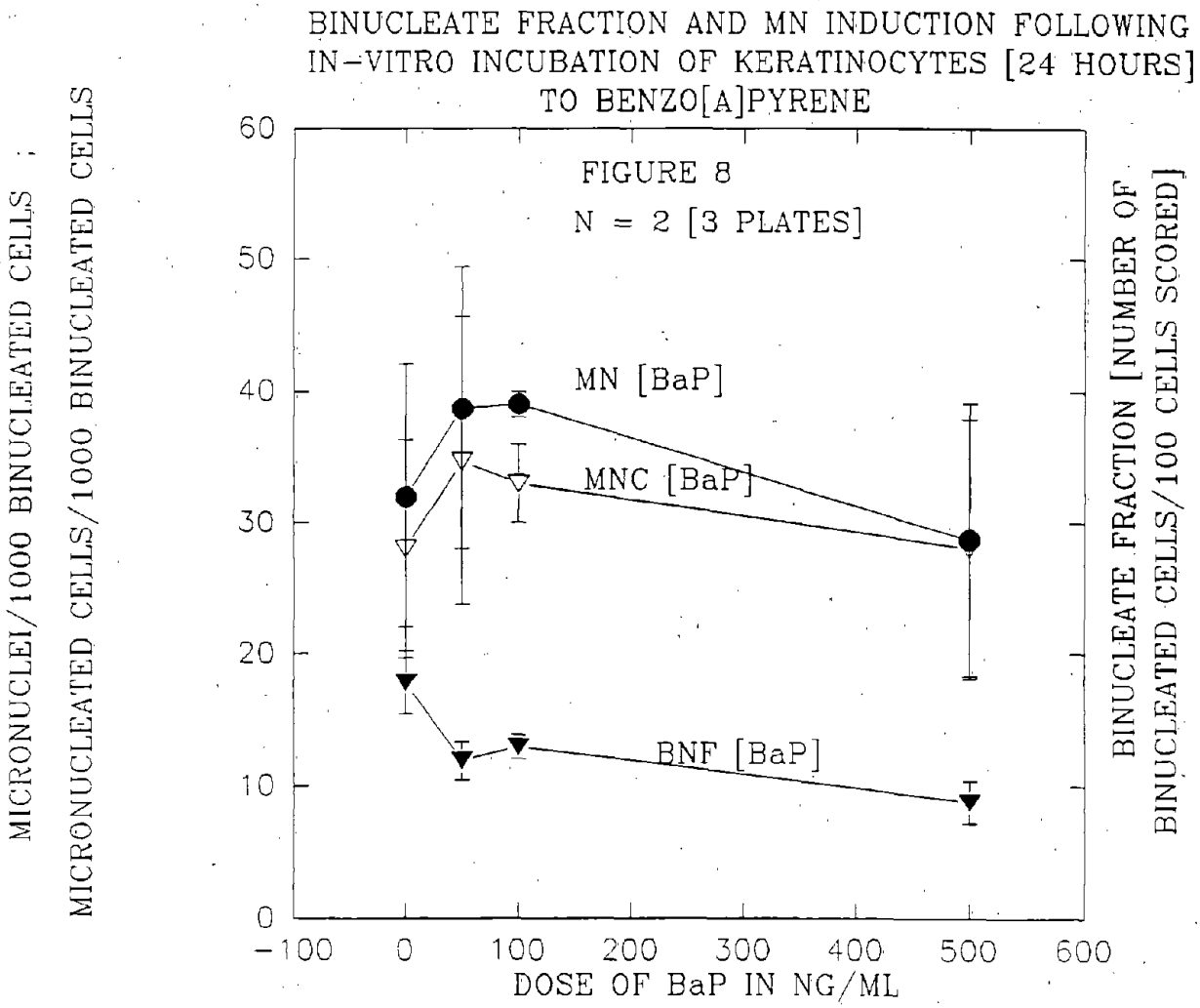
FOR MN AND DNA ADDUCT

ANALYSIS

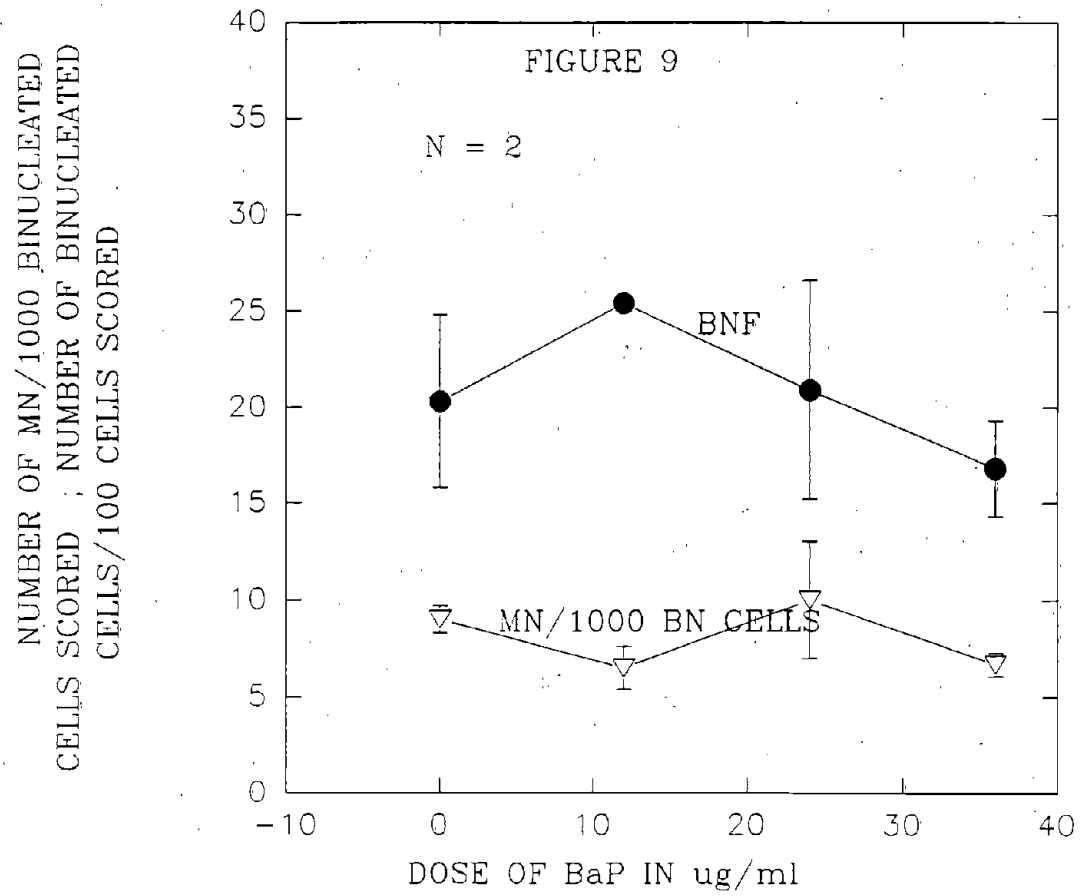


MICRONUCLEI INDUCTION FOLLOWING
IN-VITRO INCUBATION OF MOUSE KERATINOCYTES
WITH 100 NG/ML OF BaP

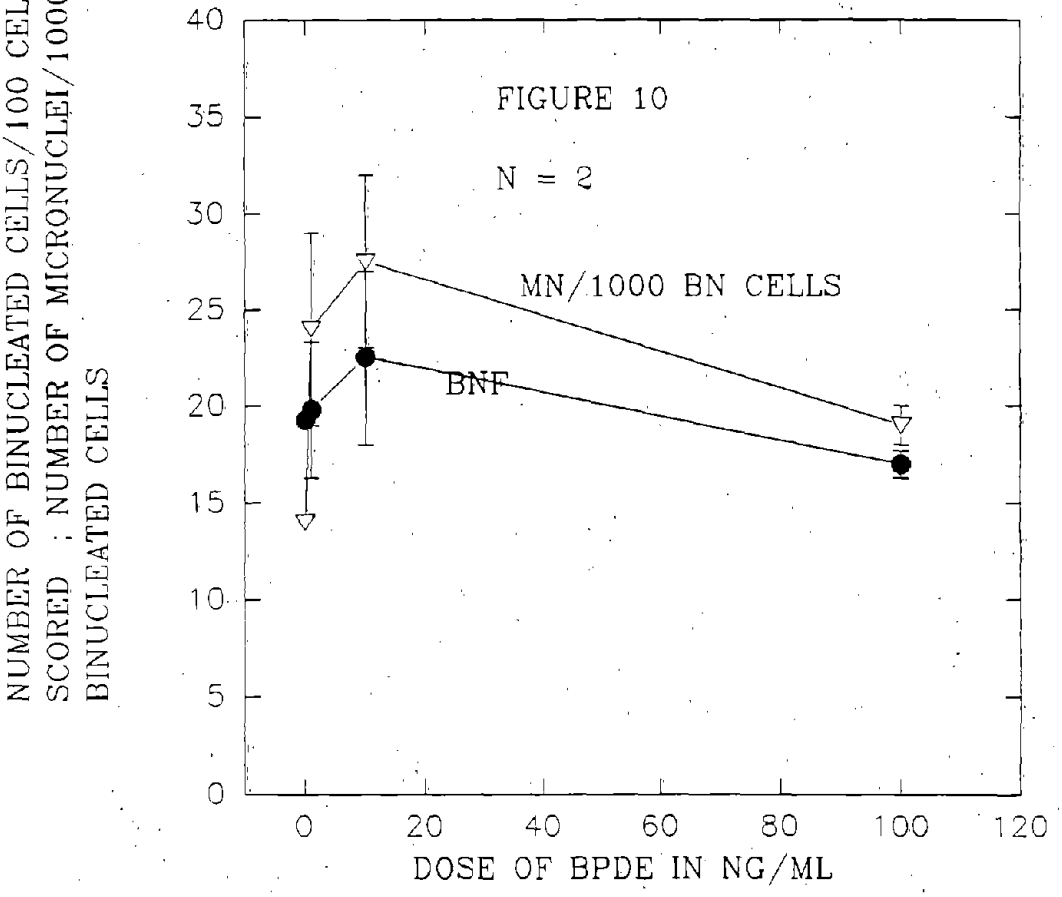


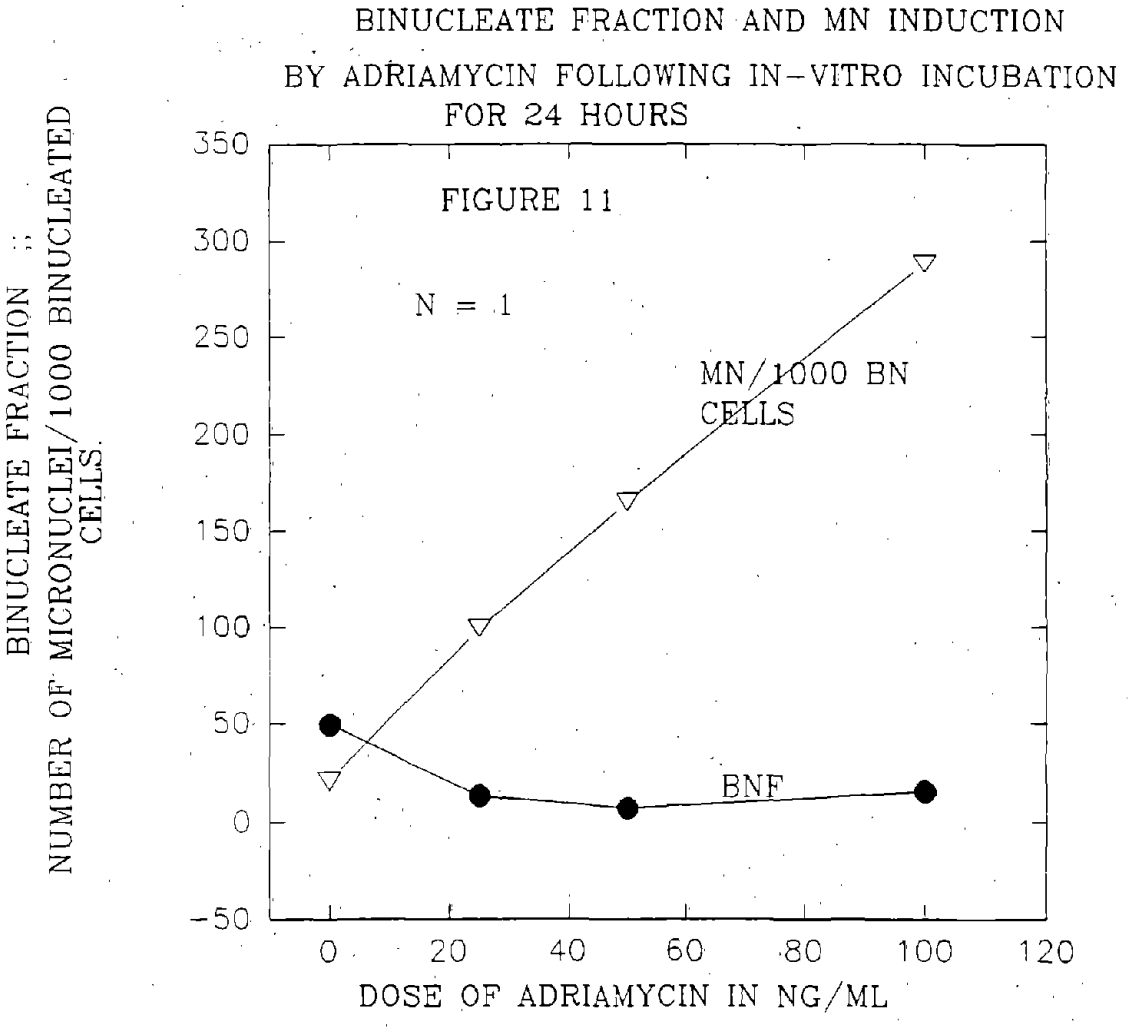

BINUCLEATE FRACTION AND MN INDUCTION FOLLOWING IN-VITRO INCUBATION OF KERATINOCYTES [24 HOURS] TO BENZO[A]PYRENE
FIGURE 8
N = 2 [3 PLATES]
MICRONUCLEI/1000 BINUCLEATED CELLS ;
MICRONUCLEATED CELLS/1000 BINUCLEATED CELLS
BINUCLEATE FRACTION [NUMBER OF BINUCLEATED CELLS/100 CELLS SCORED]
MN [BaP]
MNC [BaP]
BNF [BaP]
DOSE OF BaP IN NG/ML
0
10
20
30
40
50
60
-100
0
100
200
300
400
500
600

BINUCLEATE FRACTION AND MN INDUCTION FOLLOWING IN-VITRO INCUBATION [24 HOURS] OF BENZO[A]PYRENE



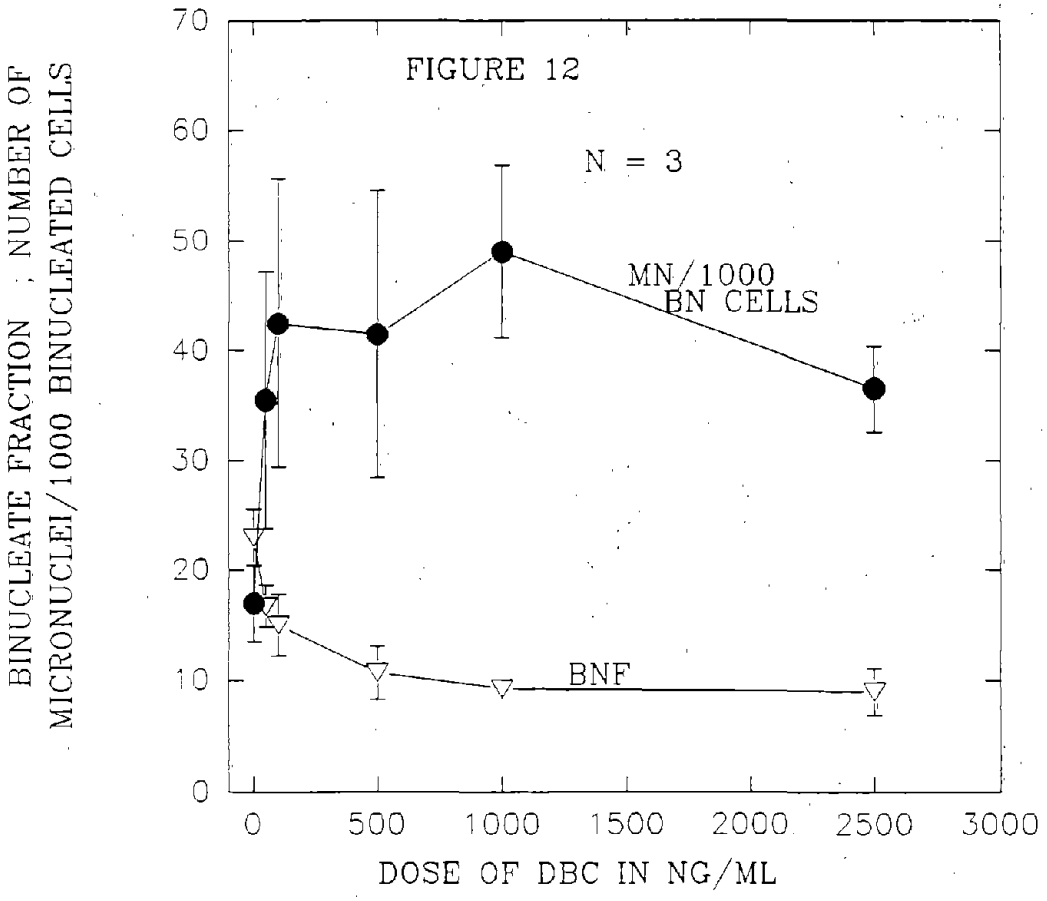
BINUCLEATE FRACTION AND MN INDUCTION
BY BENZO[A]PYRENE 7,8-DIOL, 9,10 EPOXIDE
FOLLOWING IN-VITRO INCUBATION FOR 24 HOURS




BINUCLEATE FRACTION AND MN INDUCTION
BY ADRIAMYCIN FOLLOWING IN-VITRO INCUBATION
FOR 24 HOURS
FIGURE 11
N = 1
MN/1000 BN CELLS
BNF
BINUCLEATE FRACTION ;;
NUMBER OF MICRONUCLEI/1000 BINUCLEATED CELLS.
350
300
250
200
150
100
50
0
-50
0
20
40
60
80
100
120
DOSE OF ADRIAMYCIN IN NG/ML

BINUCLEATE FRACTION AND MN INDUCTION BY
7H-DIBENZ[C,G]CARBAZOLE IN MOUSE KERATINOCYTES
FOLLOWING IN-VITRO INCUBATION FOR 24 HOURS.

FIGURE 12



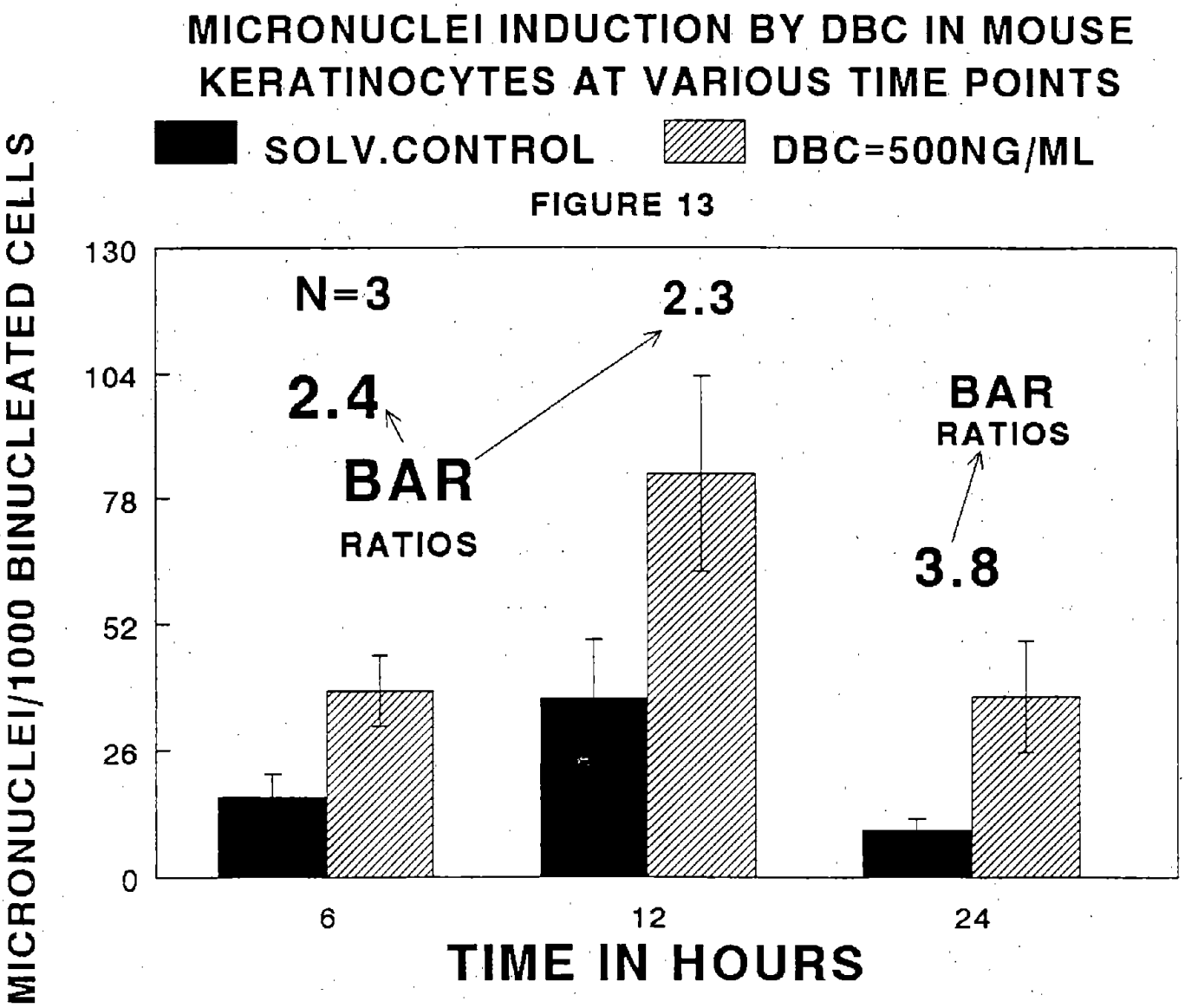
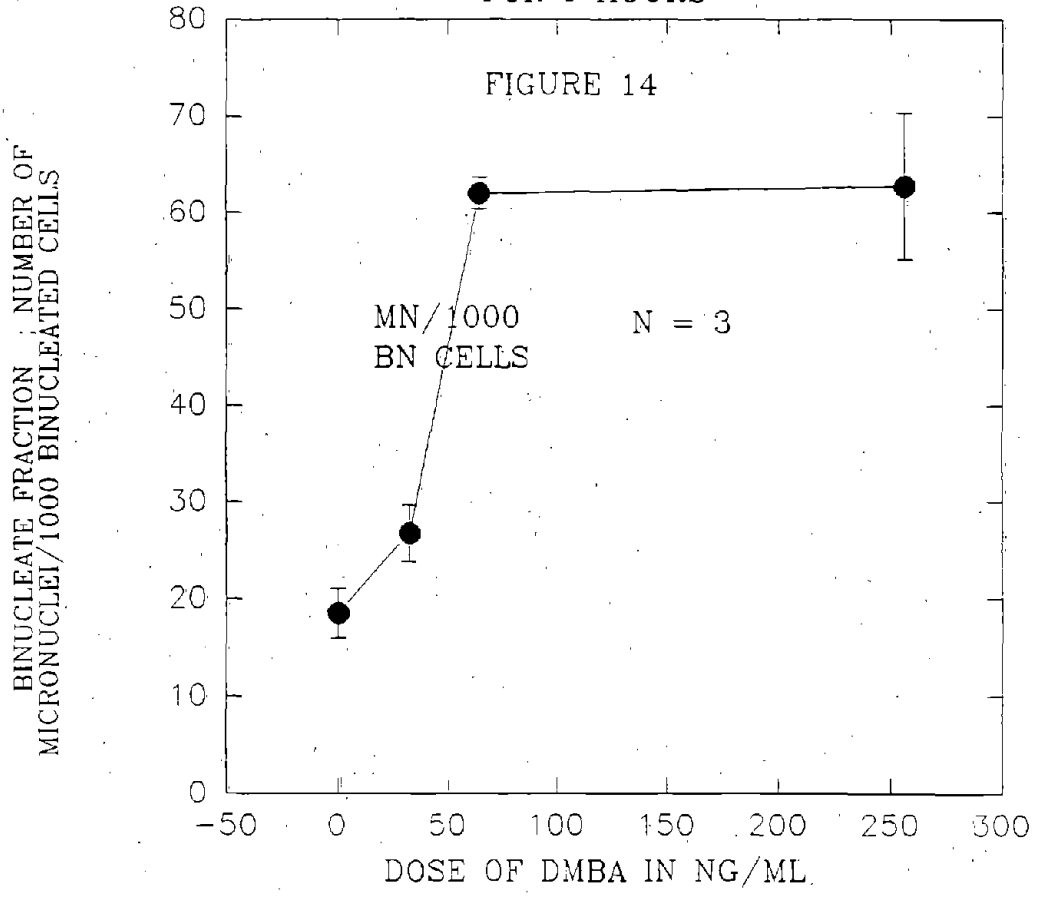

MICRONUCLEI INDUCTION BY DBC IN MOUSE KERATINOCYTES AT VARIOUS TIME POINTS
SOLV.CONTROL
DBC=500NG/ML
MICRONUCLEI/1000 BINUCLEATED CELLS
130
104
78
52
26
0
N=3
2.4
BAR
RATIOS
2.3
BAR
RATIOS
3.8
6
12
24
TIME IN HOURS

FIGURE 13

BINUCLEATE FRACTION AND MN INDUCTION BY 7,12-DIMETHYLBENZ[A]ANTHRACENE IN MOUSE KERATINOCYTES FOLLOWING IN-VITRO INCUBATION FOR 6 HOURS

FIGURE 14



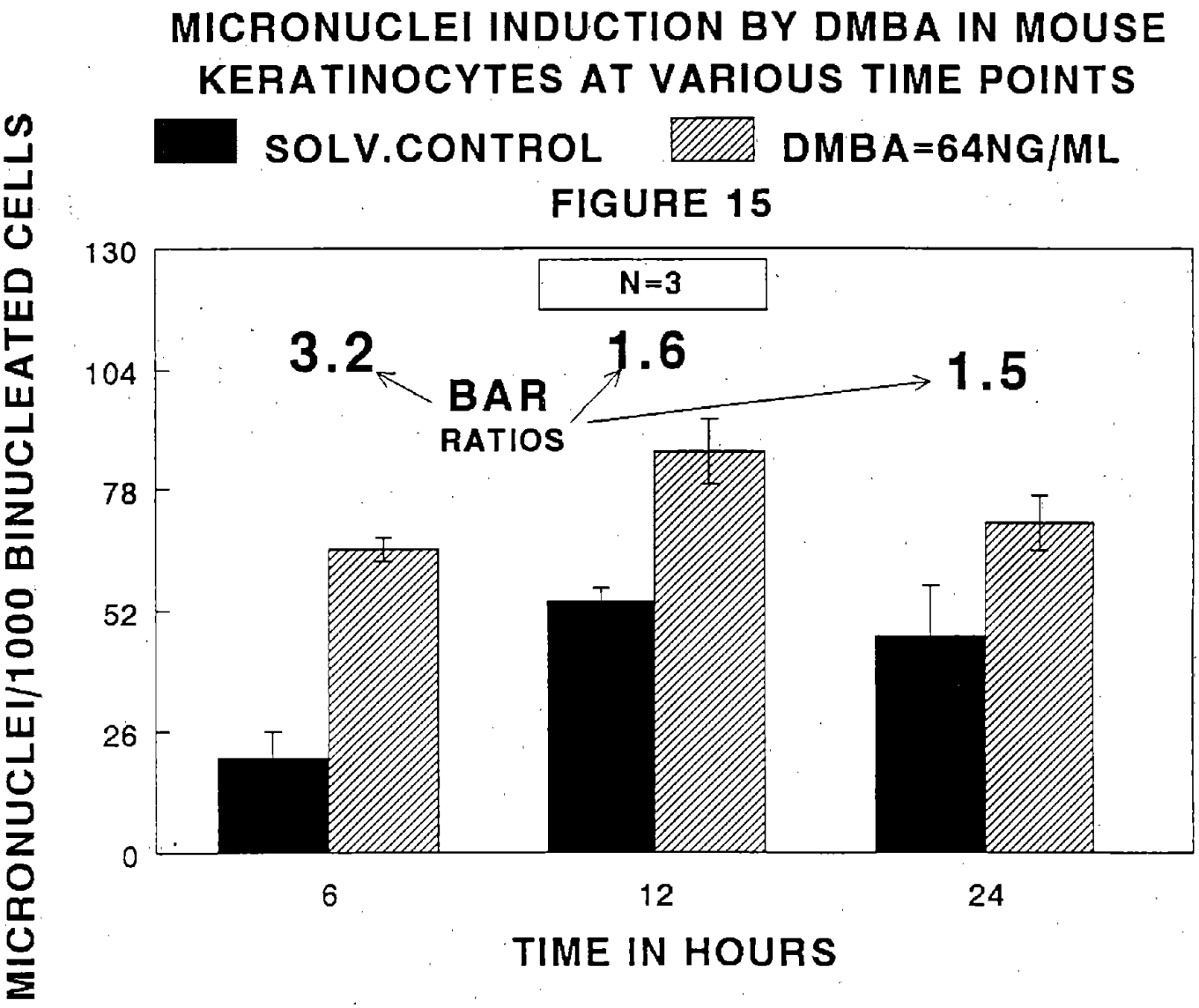
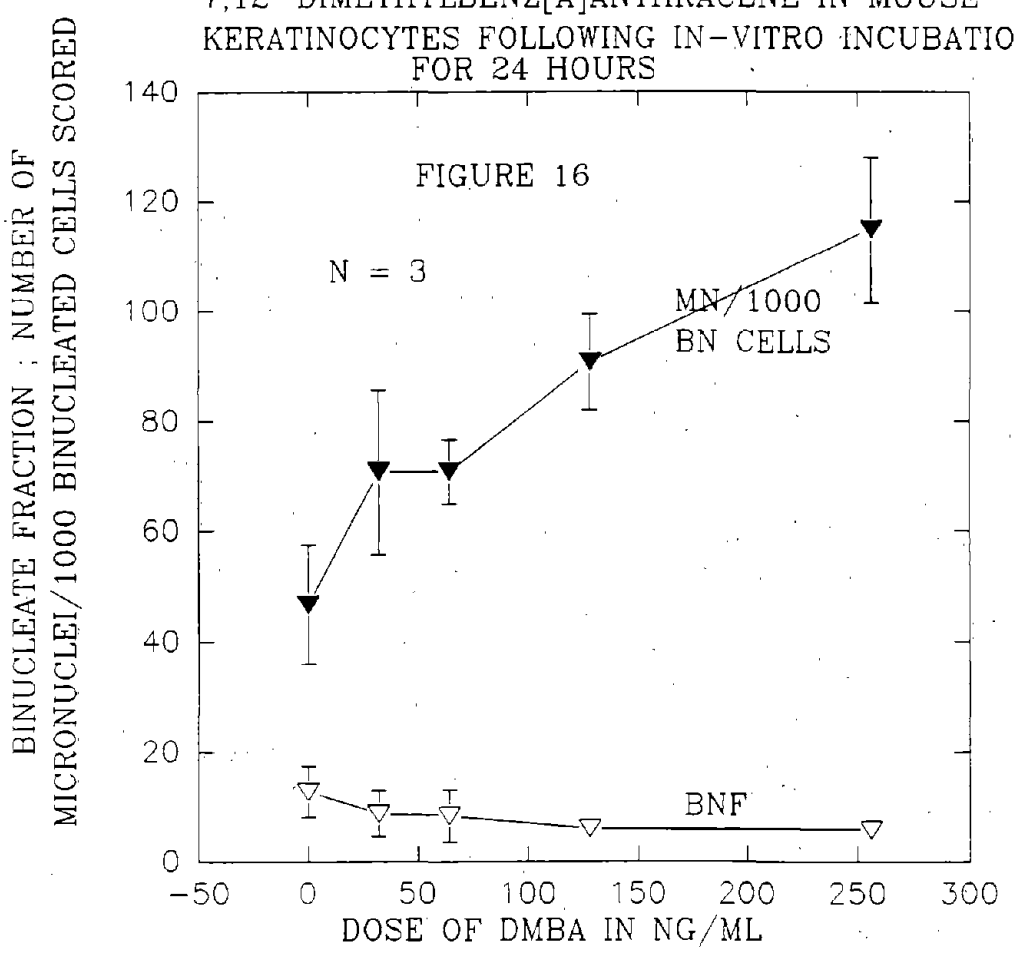

MICRONUCLEI INDUCTION BY DMBA IN MOUSE KERATINOCYTES AT VARIOUS TIME POINTS
SOLV.CONTROL
DMBA=64NG/ML
N=3
3.2
1.6
1.5
BAR RATIOS
MICRONUCLEI/1000 BINUCLEATED CELLS
130
104
78
52
26
0
6
12
24
TIME IN HOURS

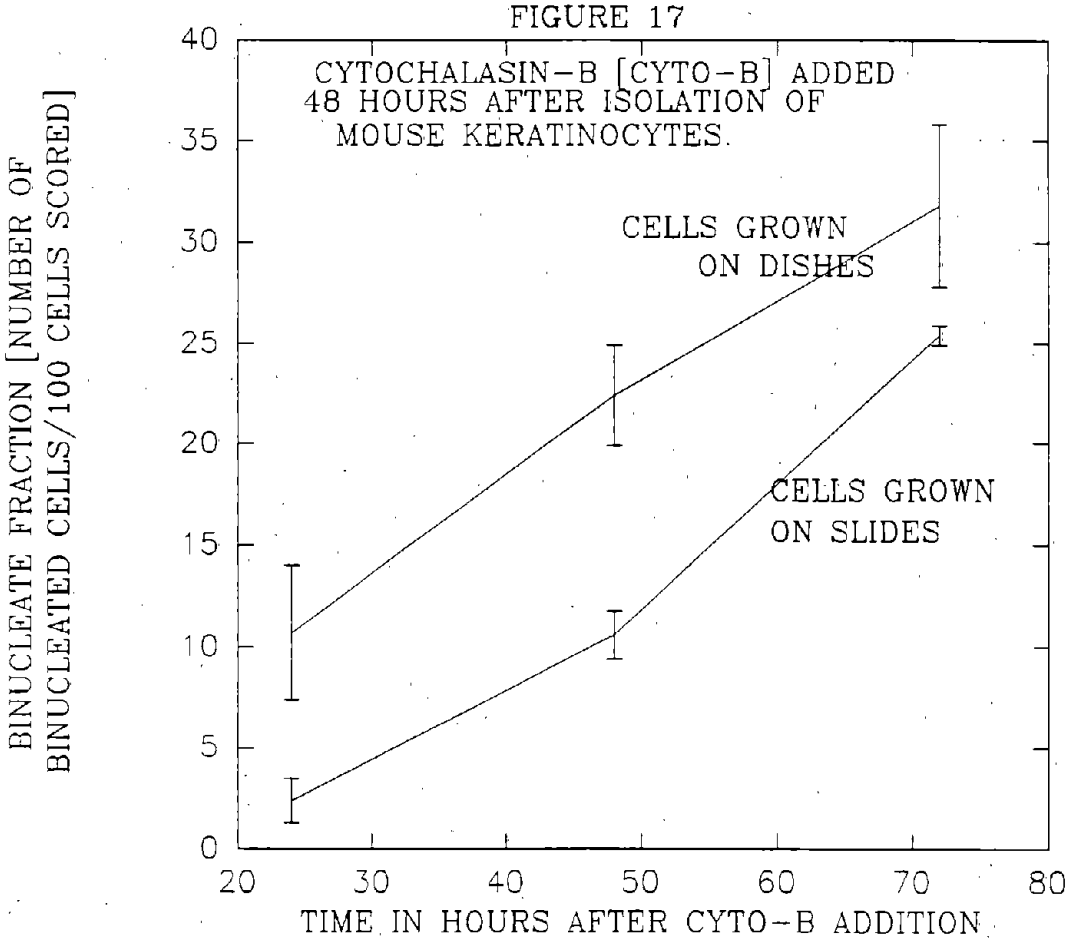
FIGURE 15

BINUCLEATE FRACTION AND MN INDUCTION BY 7,12-DIMETHYLBENZ[A]ANTHRACENE IN MOUSE KERATINOCYTES FOLLOWING IN-VITRO INCUBATION FOR 24 HOURS



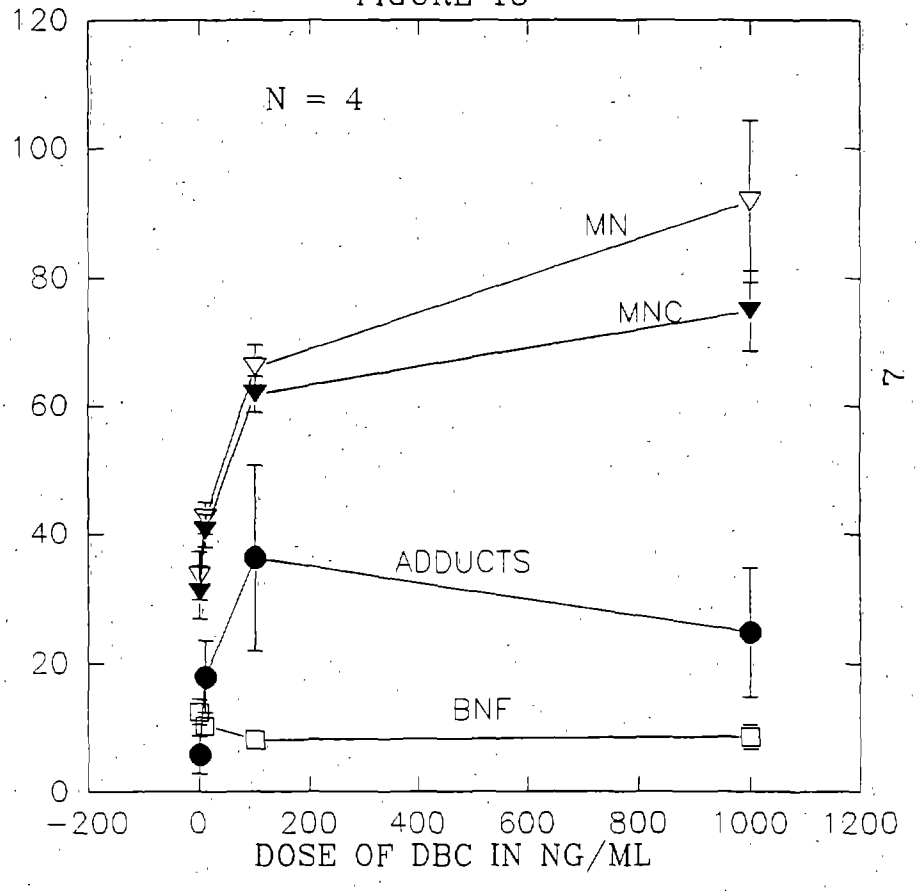
GRAPH INDICATING THAT THE BINUCLEATE FRACTION IS SIMILAR AT 72 HOURS, FOR CELLS GROWN ON SLIDES AS WELL AS CELLS GROWN ON DISHES

FIGURE 17



DATA FOR 7H-DIBENZO[C,G]CARBAZOLE
[DBC] 24 HOUR EXPOSURE

FIGURE 18

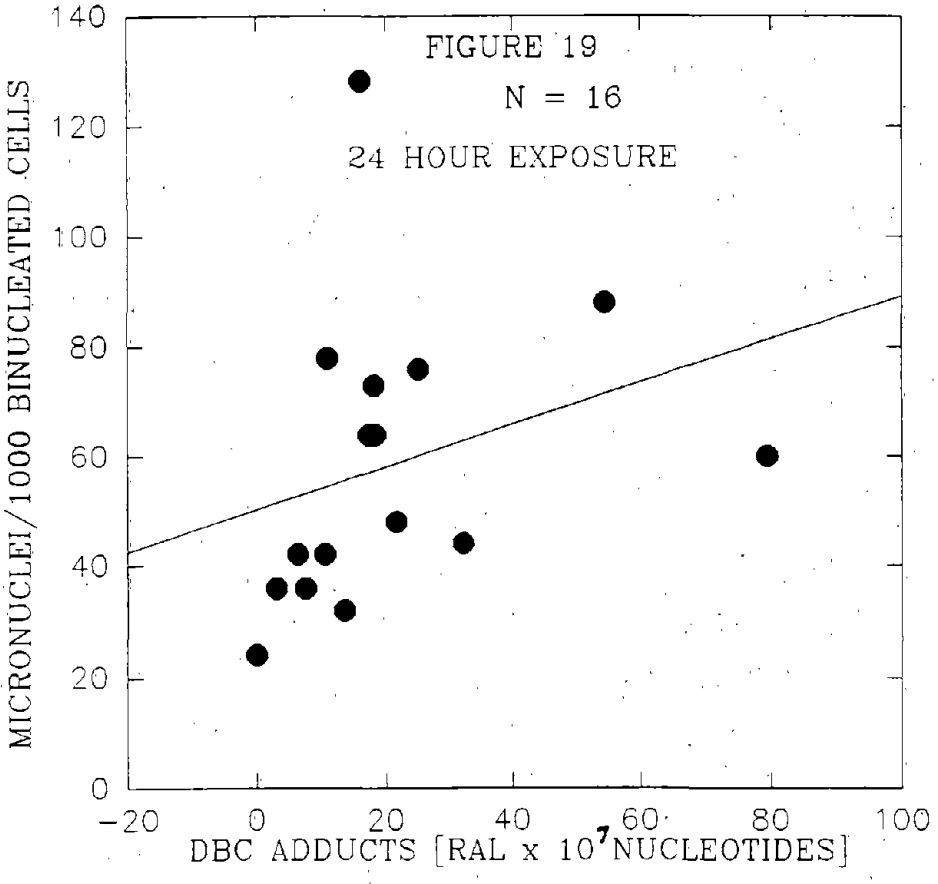


MICRONUCLEI/1000 BINUCLEATED CELLS ;
MICRONUCLEATED CELLS/1000 BINUCLEATED CELLS

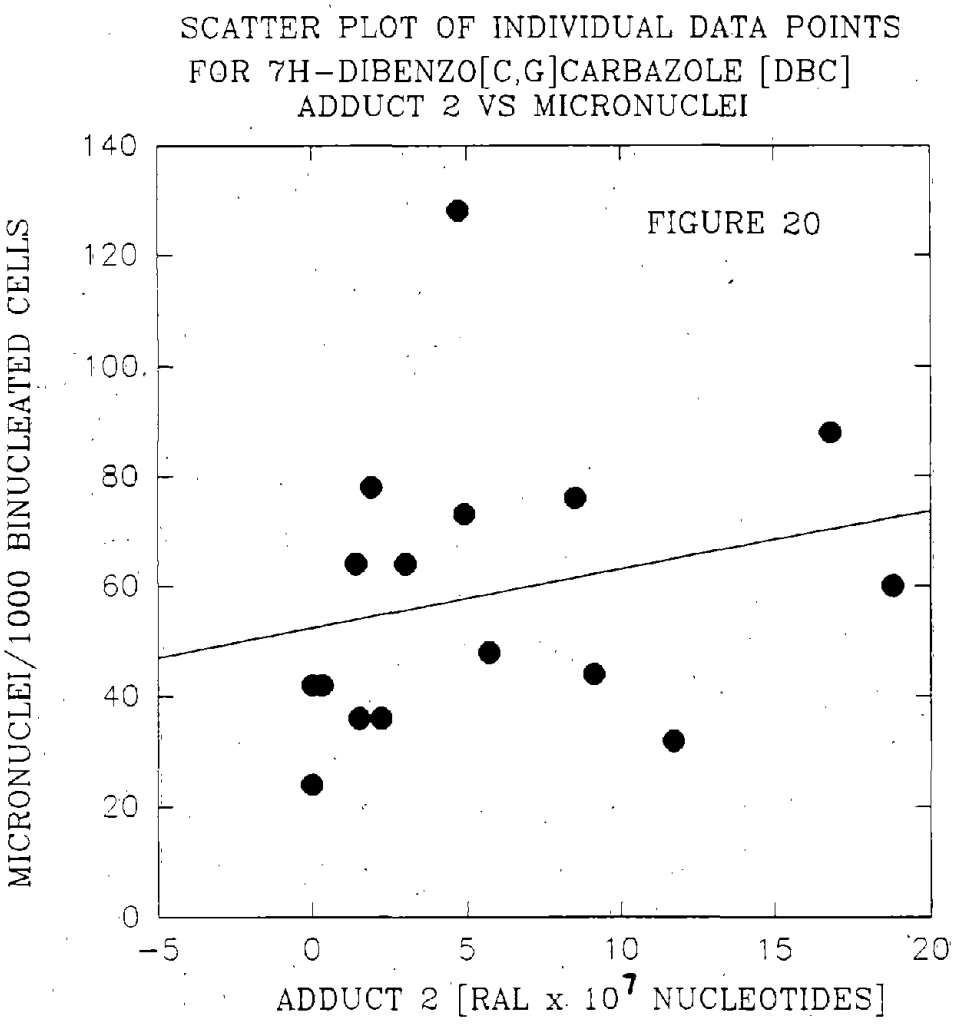
RAL x 10^7 NUCLEOTIDES ;

BINUCLEATE FRACTION [NUMBER OF BINUCLEATED CELLS/100 CELLS SCORED]

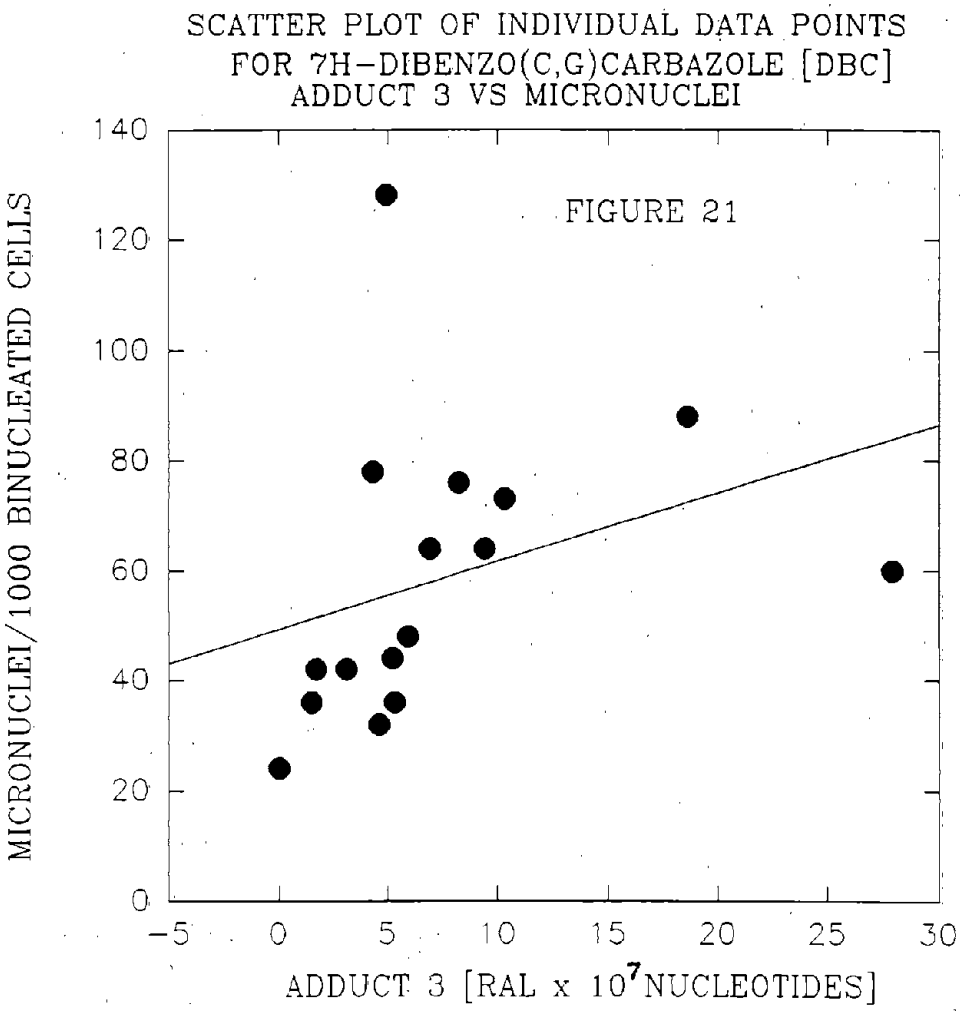
SCATTER PLOT OF INDIVIDUAL DATA POINTS FOR CONCOMITANT DETERMINATIONS OF DBC ADDUCTS AND MICRONUCLEI IN MOUSE KERATINOCYTES



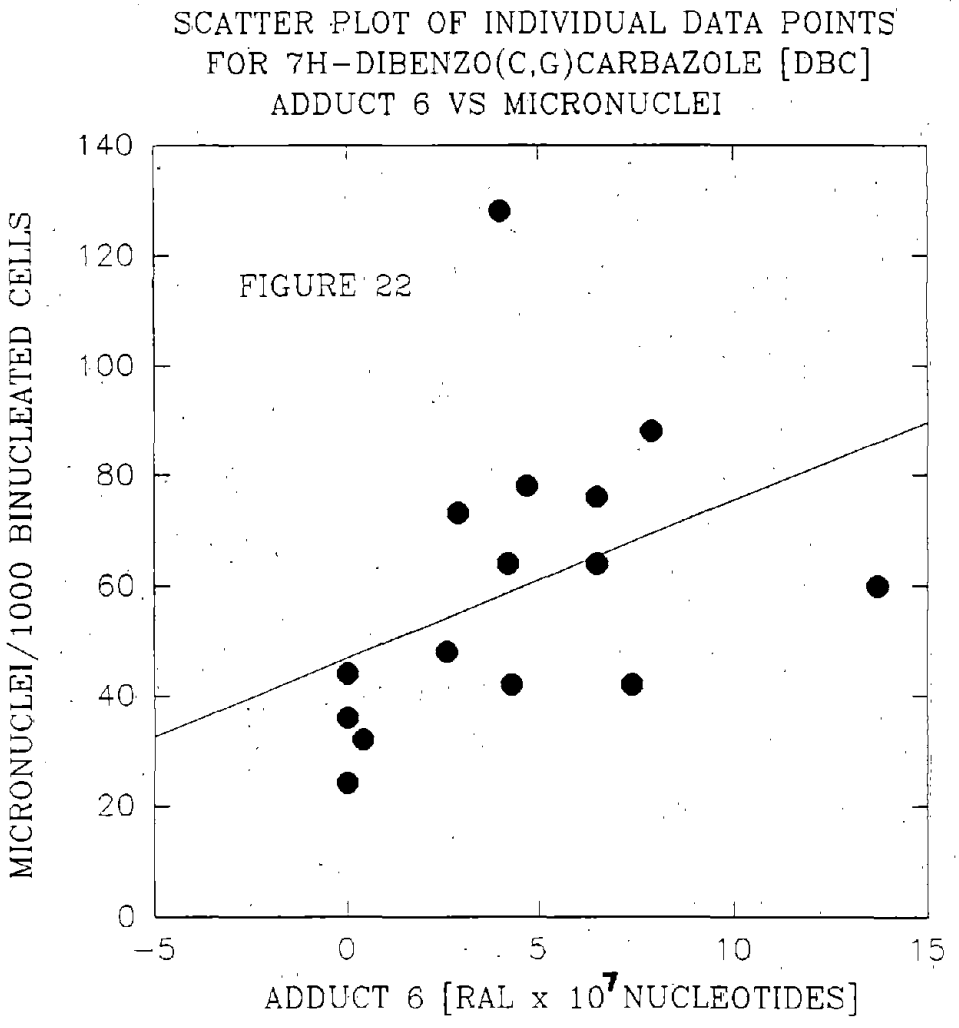
The Pearson's Correlation Coeff. is 0.6620.



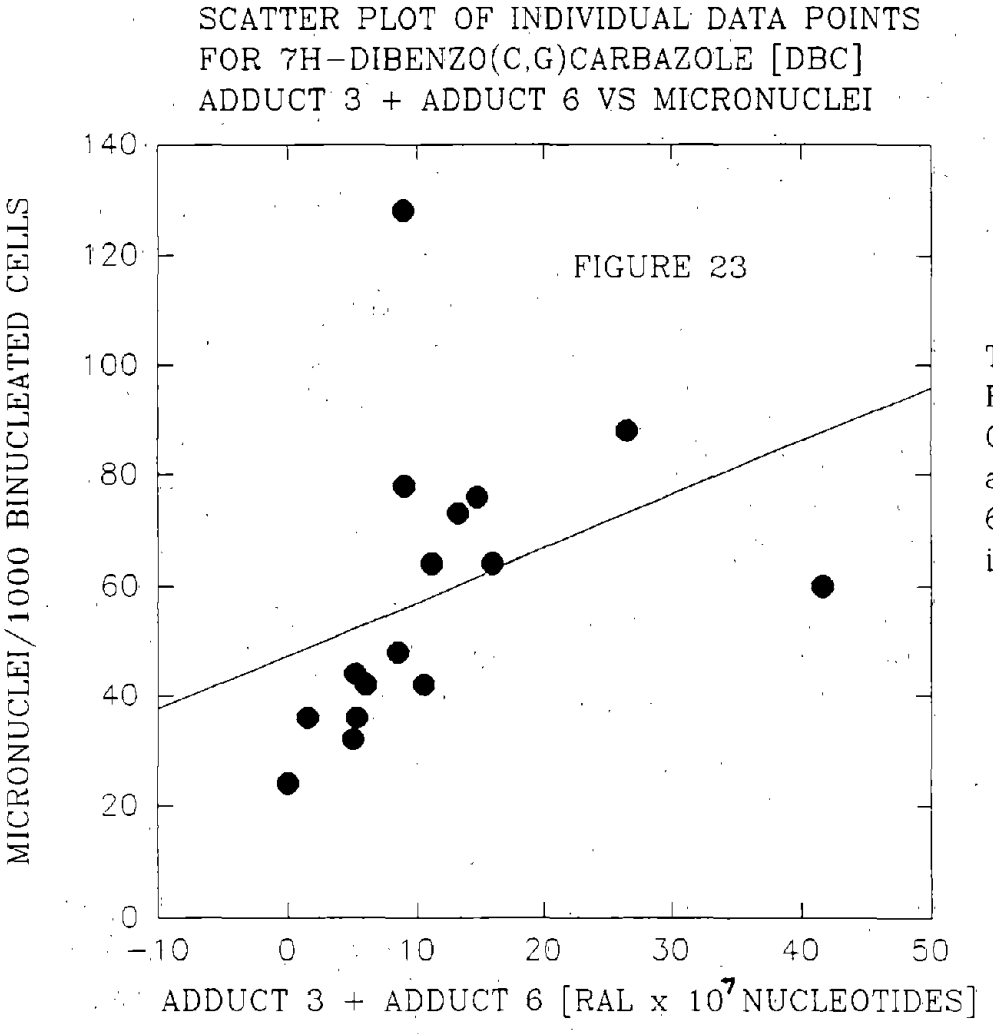
The Spearman rank correlation coefficient for adduct 2 vs micronuclei is 0.3488.



The Spearman rank correlation coefficient for adduct 3 vs micronuclei is 0.5807.



The Spearman Rank Correlation Coefficient for adduct 6 vs micronuclei is 0.5788.



The Spearman Rank Correlation Coefficient for adduct 3 + adduct 6 vs micronuclei is 0.7251.

SCATTER PLOT OF INDIVIDUAL DATA POINTS
FOR 7H-DIBENZO[C,G]CARBAZOLE [DBC]
ADDUCT 2 + ADDUCT 6 VS MICRONUCLEI.

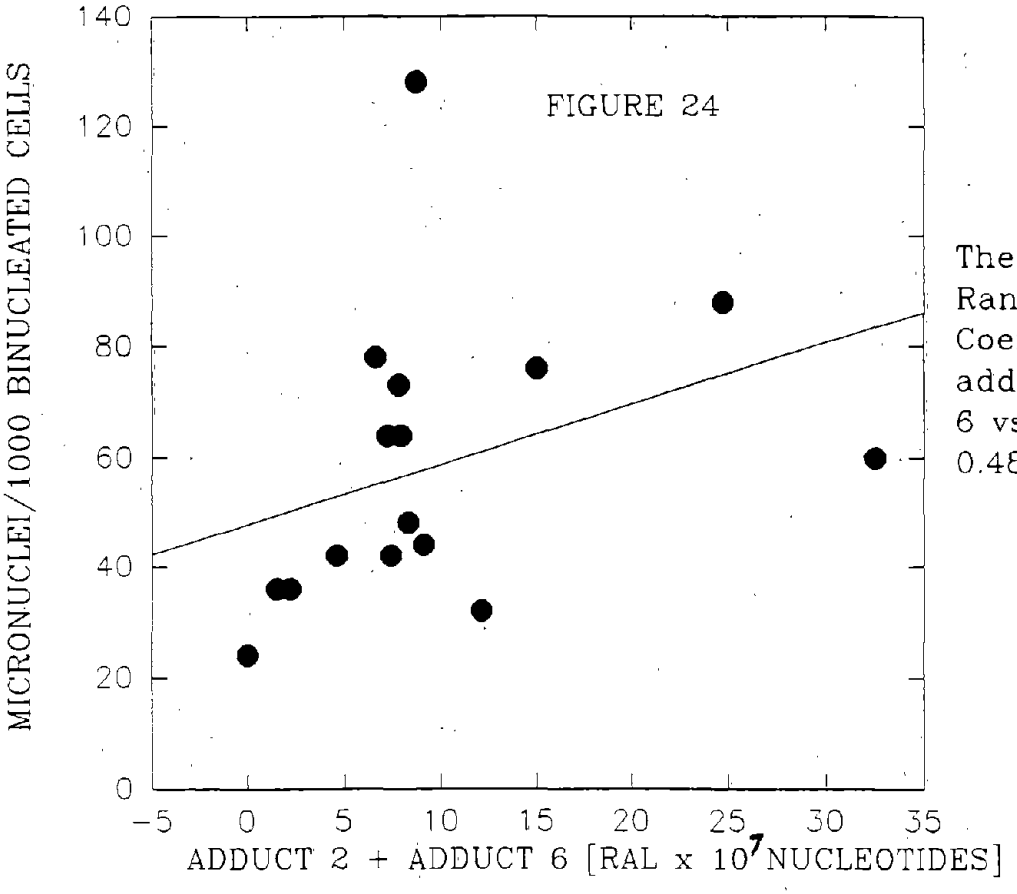
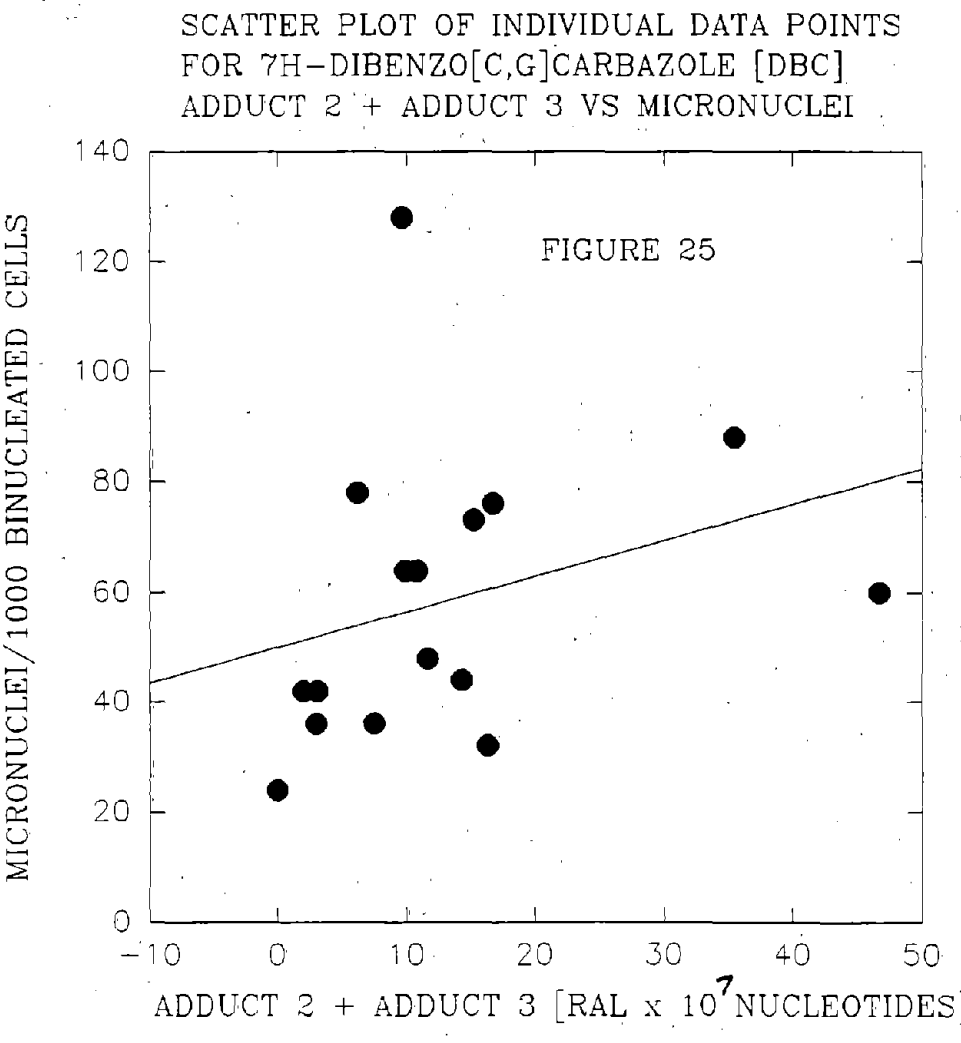
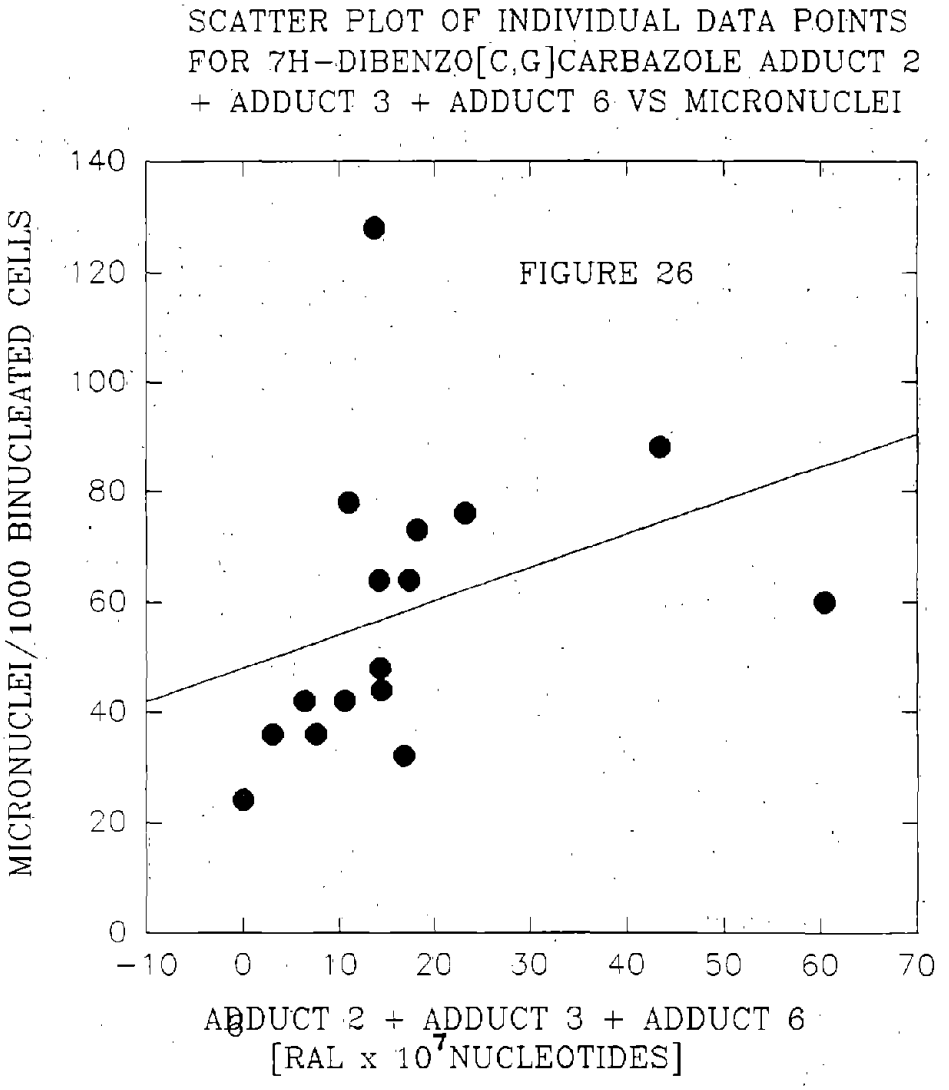


FIGURE 24

The Spearman Rank Correlation Coefficient for adduct 2 + adduct 6 vs micronuclei is 0.4819.



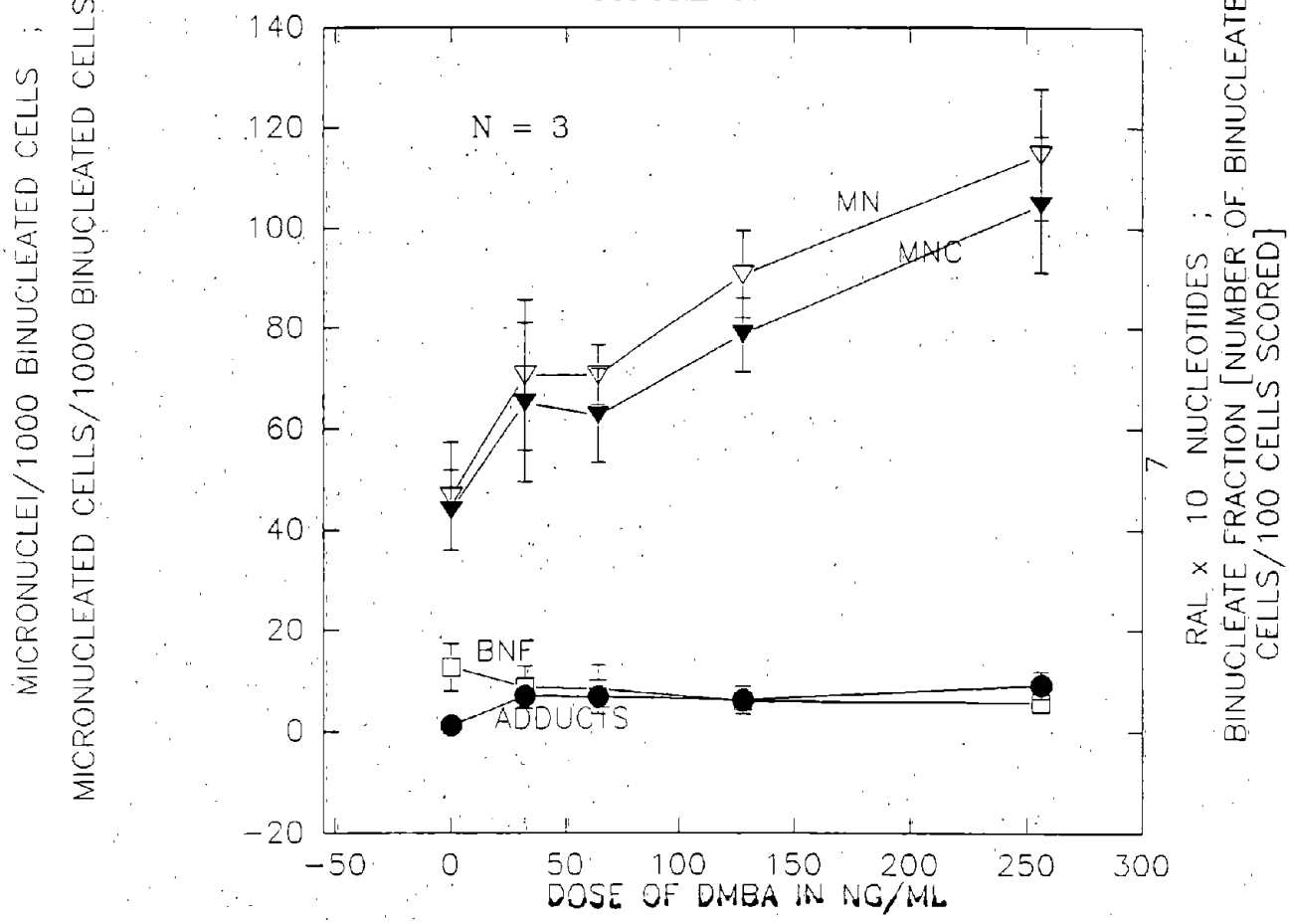
The Spearman Rank Correlation Coefficient for adduct 2 + adduct 3 vs micronuclei is 0.4363.



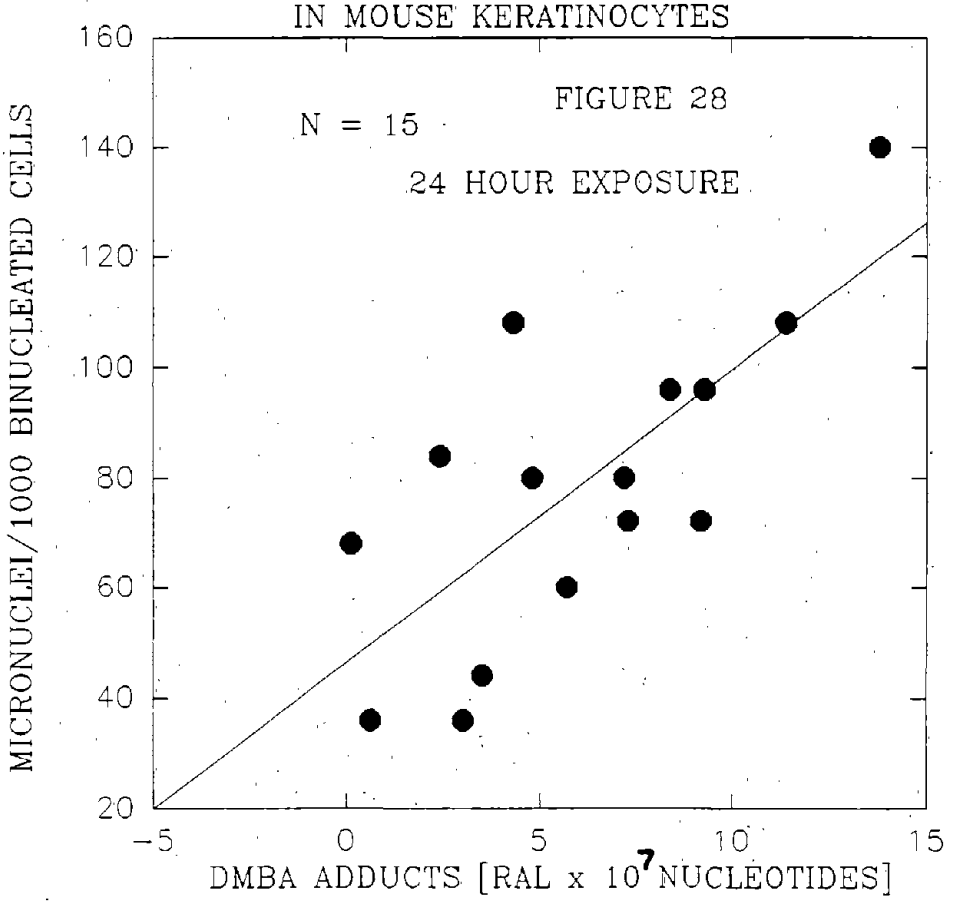
The Spearman Rank Correlation Coefficient for adduct 2 + adduct 3 + adduct 6 vs micronuclei is 0.5512.

DATA FOR 7,12-DIMETHYLBENZANTHRACENE
[DMBA] 24 HOUR EXPOSURE

FIGURE 27

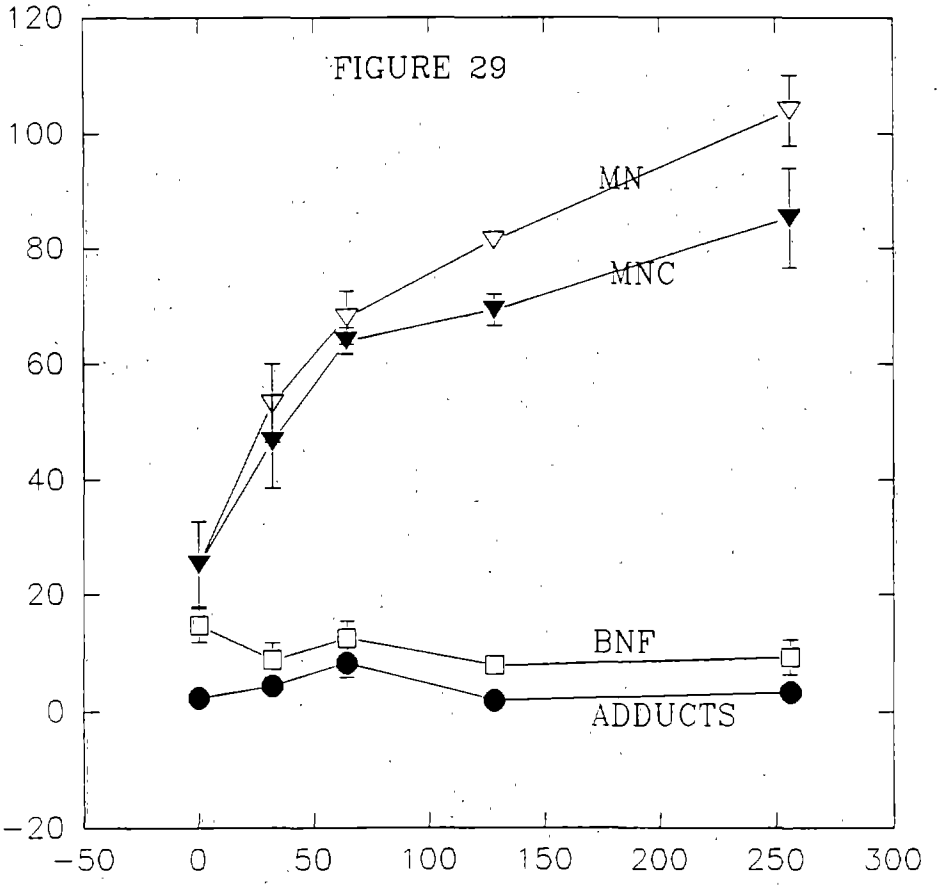


SCATTER PLOT OF INDIVIDUAL DATA POINTS
FOR CONCOMITANT DETERMINATIONS
OF DMBA ADDUCTS AND DMBA MICRONUCLEI
IN MOUSE KERATINOCYTES



The Pearson's correlation coeff. is 0.7285

DATA FOR 7,12-DIMETHYL BENZANTHRACENE
[DMBA] 6 HOUR EXPOSURE

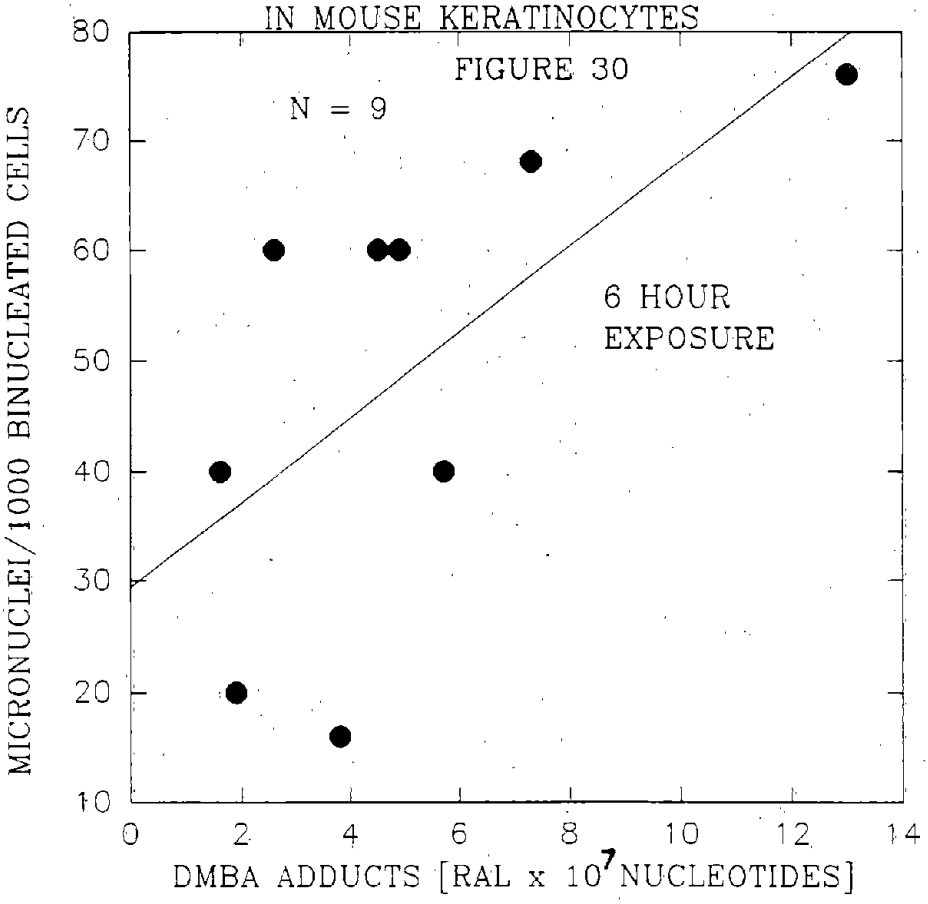


MICRONUCLEI/1000 BINUCLEATED CELLS ;
MICRONUCLEATED CELLS/1000 BINUCLEATED CELLS

RAL x 10 NUCLEOTIDES ;
BINUCLEATE FRACTION [NUMBER OF BINUCLEATED
CELLS/100 CELLS SCORED]

SCATTER PLOT OF INDIVIDUAL DATA POINTS
FOR CONCOMITANT DETERMINATIONS
OF DMBA ADDUCTS AND DMBA MICRONUCLEI
IN MOUSE KERATINOCYTES

FIGURE 30



The Spearman Rank Correlation Coeff. is 0.6810 [6 outliers deleted]

EFFECT OF 7,8-BENZOFLAVONE [I] ON DBC AND DMBA-INDUCED MICRONUCLEI

TABLE 1

DBC		DMBA	
TREATMENT		TREATMENT	
SOL. CONT.	67 +/-15	SOL. CONT.	68
[DBC] 400 ng/ml	131 +/- 19	[DMBA] 128 ng/ml	108
[DBC+I] 400 ng/ml + I (1uM/ml)	99 +/-11	[DMBA+I] 128 ng/ml + I (1uM/ml)	100
I (1uM/ml)	59 +/- 3	I (1uM/ml)	76

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