THE EFFECTS OF THREE HYDROCARBONS ON FISH CELLS IN VITRO

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CHAPTER I

INTRODUCTION

The initial basis for this study was provided by the observations of Jones et al. (1964, and unpublished data) who found that the addition of certain synthetic steroids to the water surrounding developing fish and amphibian embryos would elicit the production of hyperplasia and abnormal growths especially in the tail region. One of these steriods, 1, 3, 5(10), 16-estratetraen-3-ol (#742), was used in a series of studies to determine its cytological effects on L-fibroblasts (Bourne and Jones, 1964). It was found to have effects on cellular morphology, rates of cell division and the percentage of multinucleate cells.

The purpose of the present project was to study in vitro the cytological effects on fish cells produced by three of the most active hydrocarbons studied by Jones and Huffman (unpublished data). The three hydrocarbons used in the study were: 1, 3, 5(10), 16-estratetraen-3-ol (#742), 17α methylestra-1, 3, 5, (10)-triene-3, 17β -diol (#837), and 9, 10-dimethyl 1, 2, benzanthracene (#788-DMBA).

Cell cultures were studied on the premise that treated cells in vitro should imitate the intracellular phenomena occurring in vivo. This experimental technique is especially useful in that it allows one to

observe cells under laboratory conditions at different time periods, to compare effects of agents on cells from different organisms, to study cells of different levels of differentiation, and to eliminate in vivo changes or modifications of effects produced by cells of other organ systems.

Since tumor formation and hyperplasia are typified by a high rate of cell division, morphological changes and individual cellular abnormalities, it is felt that this direct approach is a valid experimental method for studying agents inducing abnormalities in living organisms.

Two morphologically different fish cell lines were used; a fibroblastic line from rainbow trout gonad RTG-2 (Wolf, 1962), and an epithelial line from the fathead minnow FHM (Gravell, 1965). Effects on the rates of cell division were estimated by determining the mitotic indices. It is felt that the mitotic index, as determined by large numbers of cells, is superior to either nitrogen determination or the Coulter counter methods as it allows for a thorough direct observation, and provides a more reliable count by visual elimination of dead cells and debris.

Multinucleation, often observed among malignant and anaplastic cells, and which also occurs at a higher than normal rate among cultured cells, was calculated. This aspect of the project served to detect the action of the chemicals in stimulating abnormal cell division.

CHAPTER II

LITERATURE REVIEW

One of the earliest investigators to use a form of tissue culture was an embryologist seeking answers to problems concerning embryonic regulation. Wilhelm Roux in 1885 performed an experiment which provided the foundation upon which the technology of tissue culture developed. He found that the medullary plate of a chick embryo could be maintained in warm saline for a few days (Paul, 1960).

Arnold implanted fragments of alder pith into frogs. He found that the pith would become infiltrated by leukocytes and that the leukocytes could be maintained for a few days when removed and placed in warm saline. Ljunggren in 1898 (Paul, 1960) demonstrated that cells from human skin could survive for many days in ascitic fluid.

The hanging drop method of tissue culture was contributed by Jolly in 1903. Later in 1906, Beebe and Ewing attempted to grow an "infective canine lymphosarcoma" in the blood systems of infected and uninfected dogs (Beebe, 1906). This work marked the beginning of the use of a form of tissue culture as a means for studying neoplasia.

The early controversy regarding the normal functioning of cultured cells was settled by the investigations of Harrison (1907).

Harrison explanted medullary tube tissue from frog embryos onto clots of frog lymph. These tissues survived for some weeks under aseptic conditions and development of axons occurred. This experiment is regarded as the true beginning of tissue culture (Paul, 1960).

Burrows (1910), Lewis and Lewis (1911) and Carrel (1912) contributed a great deal to tissue culture technology including the use of the plasma clot and the addition of embryo extract to the media.

From the time of Beebe and Ewing, the potentialities of tissue culture as an investigational tool in cancer research were recognized. The National Cancer Institute began to employ these methods early under the direction of Dr. Wilton R. Earle. From his laboratories came such contributions to tissue culture as propagation of cells in suspension, growth of cells directly on glass, and clone growth from a single cell.

Tissue culture has been used as a means for studying effects of chemical agents since the 1930's with the work of Brues et al (1936) on the metaphasic blockage effects produced by colchicine (Eigsti, 1955). The exact molecular nature of the colchicine effects have yet to be elucidated, but from these and other studies, a great deal of information has accumulated concerning the use of tissue culture as an investigational tool.

Creech (1940), Furukana (1960), Sacerdote (1949), Portugal (1951), Cagianut (1951), Bach (1933), Earle (1943), Goldblatt (1953),

Haddow (1939), and others have found chemical agents which would increase or inhibit cell division, cause malignant transformation, or change the morphology of cells in vitro.

Chemical #742

Jones (1964) used fish embryos, Branchydanio rerio (Hamilton) as a method of screening chemicals which affect mitosis and/or embryonic differentiation. He found 1, 3, 5, (10), 16-estratetraen-3-ol (#742) not to be cytostatistically active during early cleavage, however it did cause abnormal development and hyperplastic growth after considerable oragnogenesis had occurred. At concentrations of 2.0 parts per million (ppm) #742, all embryos tested developed necrotic tail tips, and at lower concentrations of 0.1 ppm to 0.5 ppm similar activity was noted in many specimens. Exposure of eggs and larvae of Rana pipens to concentrations of 1.0 to 5.0 ppm #742 caused bizarre epithelial growths on the tail tips.

Huffman et al (1955) found that #742 did not inhibit pituitary gonadotropin in the parabiotic rat even at dosages of 50γ. Preliminary studies done at the University of California Naval Biological Laboratory on the in vitro effects of #742 on pig kidney cells indicated that this chemical stimulated cell division (Jones, unpublished data).

Bourne and Jones (1964) studied the cytological effects of #742 on mammalian L-fibroblasts in vitro. An increase in the mitotic index from 3.64% in the control cultures to 4.50% was obtained when 0.5 ppm

#742 was added to the culture medium. 1.0 ppm increased the percentage of cells in mitosis to 6.60%; 5.0 ppm #742 increased the mitotic index to 6.06%, a slight decrease over the 1.0 ppm cultures; 10.0 ppm #742 increased the rate to 11.14%; 20.0 ppm #742 increased the rate to 13.64%; and 40.0 ppm was lethal.

Hemocytometer cell counts were made to substantiate the data obtained from the determinations of the mitotic indices. An increase in the total numbers of cells in the treated cultures over the controls was noted.

1, 3, 5, (10), 16-estratetaen-3-ol (#742) was prepared by Huffman et al (1955) by the selective benzoylation of estradiol-3, 16B to give the 3-benzoate, which was then esterified with p-toluenesulfonyl chloride to yield C_{16}-tosylate. This compound was refluxed in collidine thereby removing p-toluenesulfonic acid and effecting a double bond at C_{16}-C_{17}.

Propylene glycol, used as a solvent for the steroid, was tested to observe if any cytological effects were being produced by it. It was found that cultures containing 2% propylene glycol exhibited a mitotic rate of 7.2%, as compared with 3.64% in the controls; and cultures containing 4% propylene glycol had no cell attachment. Neukomm (1957) reported that propylene glycol had a slight effect on growth when used as a solvent for methylcholanthrene. Ruhmann and Berliner (1965) found no inhibition of growth using propylene glycol as steroid solvent at concentrations of 1.5 ppm.

Bourne and Jones (1964) also noted that marked morphogical changes were produced by #742. Cells were typed according to three morphological forms which were generally seen: type I, a large rounded cell with an abundance of cytoplasm; type II, a smaller stellate cell with definite cytoplasmic projections usually at opposite poles; and type III, a small fusiform or spindle shaped cell.

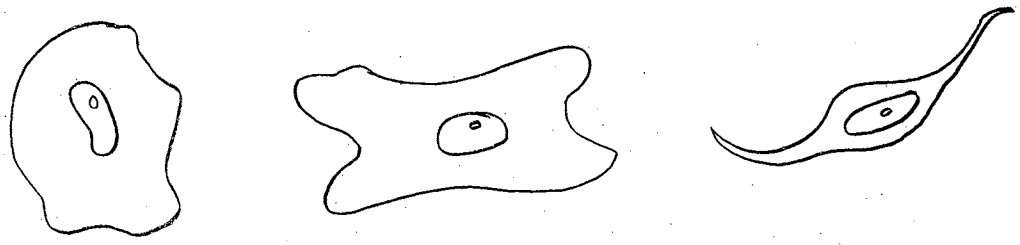

Figure 1. Three Morphological Cell Types Seen in #742 Treated L-fibroblasts

A transition from the less differentiated and anaplastic type I cell to the more typical fibroblastic fusiform type III cells was observed. This transition was directly correlated with the increase in concentration of the hydrocarbon. Propylene glycol produced only slight morphological changes toward an epitheloid type of cell at concentrations of 2.0%

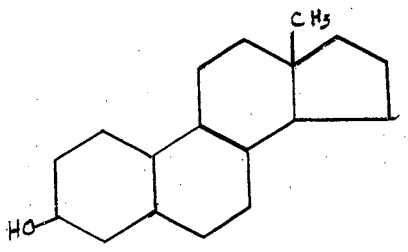

Figure 2. Structural Formula of 1, 3, 5, (10), 16-estratetraen-3-ol, Chemical #742

Chemical #837

Garner (1961) noted that 17∝methylestradiol (chemical #837) affected embryonic development of the zebra fish, Branchydanio rerio (Hamilton), in as low a concentration as 0.18 ppm when added to the water surrounding developing embryos in early cleavage. Especially the tail and gut were susceptible to damage by the chemical. At concentrations of 0.5 and 1.0 ppm, little development occurred in the early embryonic stages.

It was noted that the cleavage lines present at the time of exposure underwent fading, and thirty minutes after exposure, cells of the early cleavage embryos appeared indistinct as separate cells. Tests run on 24 hour embryos, which had already undergone considerable organogenesis, showed that low concentrations of 0.56 ppm and 1.0 ppm to have little or no effect. Higher concentrations of 1.8, 3.2, 5.6 and 10.0 ppm immediately impaired cell division.

Cytological examination indicated the chemical had the effect of producing a stoppage of mitosis in whatever stage the cell was in, with a preference for metaphase (Garner, p. 18). Nuclear membranes of prophase cells were observed to break down with subsequent termination of all mitotic activity. If the cell was in telophase when the chemical was applied, the nucleus underwent degeneration. The coalescing of cells appeared to give rise to multinucleation.

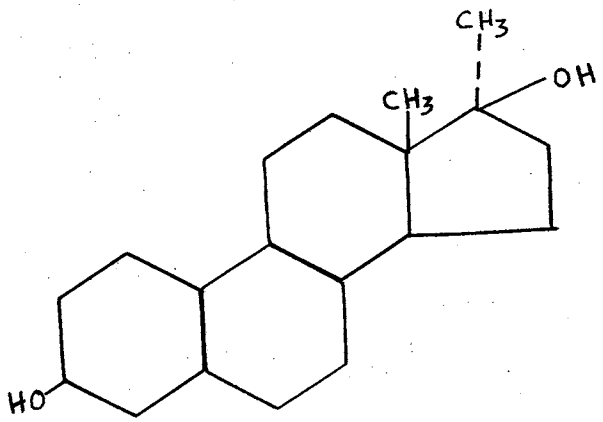

Figure 3. Structural Formula of 17α methylestra-1, 3, 5(10)-triene-3, 17β diol, Chemical #837

Chemical #788 (DMBA)

9, 10 dimethyl 1, 2, benzanthracene (DMBA) has long been known as a potent carcinogen (Badger, 1940; Bachmann, 1938; Brandbury, 1941; Law, 1940). Haddow (1939) reported DMBA to have an inhibitory action on the growth of spontaneous cancers. In his studies on the influence of polycyclic hydrocarbons on growth rates of primary sarcomas, he found all induced sarcomas to be more resistant to the growth inhibitory action of carcinogenic hydrocarbons than were the transplanted and spontaneous sarcomas (Haddow, 1938).

More recently, Starikova and Vasiliev (1962) found normal primary cultures from rats to be strongly inhibited by very low concentrations (2.5×10^{-6} mg/ml) of DMBA. Such concentrations reduced the mitotic activity by three to five times. Higher concentrations of DMBA (2.5×10^{-3}) diminished the mitotic rate to zero. This decrease was

clearly demonstrated not to be due to an alteration of the mitotic time.

DMBA-induced rat sarcoma cell cultures treated with DMBA were only slightly inhibited. Cell cultures of sarcomas induced by other methods were also resistant to DMBA. Even Sarcoma 45, a transplantable rat sarcoma induced by DMBA over a decade ago, was still insensitive to inhibition by DMBA.

Vasiliev and Starikova demonstrated DMBA-induced sarcomas were, however, sensitive to the mitotic inhibitory effects of the cancer chemotherapeutic sarcolysin. However, sarcolysin had no effect on normal fibroblasts. These findings have been substantiated by other laboratories (Berwald and Sachs, 1963; Alfred, 1964).

Diamond et al. (1967) studied the mechanism of cytotoxicity and the possible relationships which may exist between cytotoxicity and carcinogenicity. By means of randomly labeled DMBA-^{3}H and fluorescence microscopy, binding of DMBA to sensitive and resistant cells was compared. Autoradiography and fluorescence microscopy revealed DMBA to be concentrated in the cytoplasm of both resistant and sensitive cells as well as in their nuclei; differences, however, were found in the comparison of amounts of the hydrocarbon that remained after fixation in lipid solvents. Sensitive cells retained significant quantities of the labeled material; resistant cells did not. Bound hydrocarbon was found in both the cytoplasm and the nucleus of sensitive cells. Radioactivity assays of cellular DNA and RNA demonstrated the normal

cells bound 10 to 50 times as much DMBA-^{3}H as did the malignant cells.

Tests performed by Jones et al. on DMBA by adding the chemical to the water surrounding developing Zebra fish embryos in varying concentrations, revealed DMBA at concentrations of 0.56 ppm to cause tail necrosis, tumor-like growths, an enlarged pericardium and a shortened body. Cells and their nuclei on the surfaces of the test embryos were enlarged and had a granular appearance. Some parts of the developing embryos' bodies seemed to be growing at a more rapid rate than other parts. These abnormalities were apparent within 24 hours exposure time as compared with 48 hours for the appearance of effects produced by #742. At 96 hours' exposure time to 0.56 ppm DMBA, effects produced were quite similar to those of #742. At 5.0 ppm DMBA stopped embryonic development completely, and after exposure for 24 hours all of the test embryos were killed. At a concentration of 3.2 ppm DMBA, two embryos out of ten developed no posterior parts. (Jones, unpublished data).

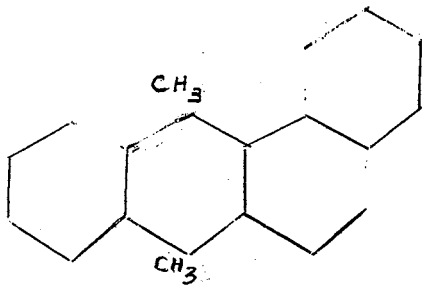

Figure 4. Structural Formula of 9, 10 Dimethyl 1, 2, Benzanthracene, DMBA #788

CHAPTER III

MATERIALS AND METHODS

Cell Cultures

Two morphologically different fish cell lines were used, rainbow trout gonad (RTG-2), a fibroblastic line, and fathead minnow (FHM), an epithelial line.

Line RTG-2, the first permanent fish cell line, was established by Wolf and Quimby (1960) from rainbow trout gonadal cells (Salmo gairdneri) obtained by primary cultivation of pooled gonads of male and female yearling fish. Original cultures were established by cold trypsinization and maintained in a cord serum medium at 19°C. These original cultures contained both epitheloid and fibroblast-like cells.

The line was subcultured in a variety of media, supplemented with fetal calf serum, which were designed for homeothermic animal cells. Antibiotics have been used in as high a concentration as 400 units of penicillin-streptomycin per milliliter, and cultures were routinely grown in antibiotic free media. Incubation temperature was found to be of critical importance in the maintainence of fish cell cultures. RTG-2 cells have been grown at temperatures ranging from 4°C to 26°C, with the latter being the upper limit of heat tolerance. (Wolf, 1962).

Several methods of subculturing have been used, as the "policing" method, ten minute cold-digestion with 0.25% trypsin and ten minute cold-dispersion with sodium versenate (20 mg/100 milliliters). Of the three methods, ten minute cold dispersion with sodium versenate has been most satisfactory since the membranes of RTG-2 cells seem to be highly susceptible to digestion by trypsin. Recently, a mixture of trypsin-versene has been found to be an excellent dispersal method, and is satisfactory for rendering single cell suspensions for cell counting using the Coulter counter (Wolf, personal communication).

The in vitro modal chromsome number of line RTG-2 was found to be 59 with a range from 49 to 71. The diploid number for Salmo gairdneri is 60 (Wolf, 1962). The line is highly susceptible to the virus of infectious pancreatic necrosis of trout.

Line FHM was established by Gravell and Malsberger (1965) from the fathead minnow (Pimephales promelas). In this case the tissue posterior to the anus was excised and cells dispersed with trypsin (0.25%) for four hours at 4°C. The primary cells were described as being epithelial cells. These cultures have been maintained in both Eagle's basal and minimal essential media supplemented with fetal calf serum. Maximum rate of growth was found to occur at 34°C, and minimal growth at 4°C. The diploid chromosome number obtained from in vitro cells was found to be 50 ± 2; however the in vivo chromosome number has not been reported for the northern fathead minnow (Gravell, 1965). Determinations of chromosome numbers some time

after the line was established indicate the number is constant. The line will support growth of a number of viruses i. e. infectious pancreatic necrosis virus of trout, lip tumor virus of the brown bullhead, and also the mammalian ECHO-11 virus. The FHM line is a hardy, easily-maintained and rapidly growing cell line.

Cell lines FHM and RTG-2 were obtained from Dr. Ken Wolf at the Eastern Fish Disease Laboratory, Leetown, West Virginia and brought to this laboratory in June, 1964. The cell lines have been maintained in serial cultivation since that data. All cultures have been grown in minimal essential media containing 10% fetal calf serum, 1% L-glutamine (stock concentration 200mM/ml) and 200 units of penicillin-streptomycin per milliliter. All tissue culture media, reagents, antibiotics and sera were obtained from Microbiological Associates, Bethesda, Maryland. The pH was maintained at 7.0 to 7.3 by gassing the cultures with 5% carbon dioxide in air.

The cultures have been incubated at 21°C ± 1°C, and reserve cultures at 4° - 6°C. Initially, cultures were grown in pyrex tissue culture tubes (Curtin) containing 1.5 ml media and placed in the incubator on 5° angle racks. When larger numbers of cells were needed, 8 ounce "milk dilution" bottles with autoclavable caps (Curtin) were used with ten ml of media. The larger containers were preferable since they greatly reduced the amount of time required to cultivate large quantities of cells.

Subcultures were made when the monolayer was estimated to be approximately 80% confluent. It was found that the cultures would grow more rapidly and maintain a healthy appearance if they were subcultured while in an active growth phase. All operations with open cultures were carried out in a 22" X 32" hood with a plexiglass cover, and equipped with a vent and touchomatic burner. The interior of the hood was washed weekly with a 5% chlorox solution, thoroughly rinsed with water, and air dried. The interior of the hood was wiped down before each use with 70% isopropyl alcohol.

RTG-2 cell cultures were found to be extremely sensitive to exposure to temperatures over 25°C even for very short periods of time. For this reason, flaming of bottle necks, pipette tips, etc., was abandonned. Contamination was kept to a minimum by thoroughly washing the hands with 80% ethanol, or by using sterile rubber gloves.

Subculturing was done by a ten minute cold dispersion with versene followed by "neutralization" with the old medium. Versene was prepared as follows: 8.0g NaCl, 0.2 g KH_2PO_4, 0.2 g KCl, 1.15 g Na_2HPO_4, 0.2 g ethylenediaminetetraacetic acid; the ingredients were dissolved in 100 milliliters distilled deionized water, then filter sterilized. The pH was adjusted to 7.3.

After the monolayer had been dispersed with versene and "neutralized", the media and cells were pipetted into centrifuge tubes and centrifuged at 1000 rpm for ten minutes at 4°C. Old media and

versene were then decanted and the pellet resuspended in fresh medium. Aliquots of the suspended cells were then distributed to sterile culture bottles.

The FHM line was easily dispersed with versene, but the RTG-2 line had a tendency to form clumps. Even with vigorous pipetting with nine inch Pasteur pipettes, it was difficult to disperse the cells adequately. In some instances clumps were simply removed from the cultures and discarded rather than attempting to break them up.

Water used in the preparation of basic salt solutions, rinsing glassware, etc., was obtained from a Barnstead water still (catalog number SMH-5), and run through a Barnstead Bantum deionizer (Barnstead Still and Sterilizer Company, Boston, Massachusetts). Water was collected in ten-gallon polyethylene jugs.

Tissue culture glassware and utensils were washed according to the methods of Wolf and Quimby (personal communication) in order to maintain the cells under identical laboratory procedures. All glassware, rubber stoppers, etc., were soaked in a Calgon-Metasilicate solution ("C&M") prepared in the following manner: 26.5 g of Calgon (Calgon Water Conditioning Company, Pittsburgh, Pennsylvania) and 238.0 g of sodium metasilicate (Fisher Scientific Company) were dissolved in 2,500 milliliters of deionized water and diluted 1:100 before use.

Glassware was washed twice under warm tap water with a brush, placed in fresh "C&M" solution and boiled for 20 minutes. This was followed by a thorough rinsing in warm tap water. Each piece was inspected for cleanliness before three final rinses in distilled, deionized water. Equipment was air dried.

Pipettes were placed in "C&M" solution immediately after use, soaked overnight, and thoroughly washed in hot tap water in a Nalgene automatic washer (Nalge Company, Rochester, New York). Pipettes were boiled for 20 minutes in fresh "C&M" solution, rinsed in hot tap water, then rinsed three times in distilled deionized water.

When necessary, pipettes were soaked in dilute nitric acid solution before washing, and were always thoroughly rinsed before going through the washing procedure. Pipettes were cotton-plugged and placed in pipette cans before sterilizing. All glassware and utensils were sterilized in a Castle autoclave (model 99-C, Wilmont Castle Company, Rochester, New York) for at least fifteen minutes at 16 pounds pressure per square inch at 270° F. Liquid materials which could not be heat-sterilized were filter-sterilized with a Millipore filtering apparatus (catalog number XX10 047 00) using a final filter of 0.45 μ pore size.

Experimental cultures were set up using the same subculturing techniques as were used for the stock cultures. All cells were grown directly on coverslips in Leighton tubes (Microbiological Associates).

A large number of control cultures were grown before any experimentation was carried out in order to determine if standardized procedures, such as uniform media, 72 hours incubation time, pH, etc., would provide cultures having a rather uniform mitotic rate. After it was determined that the mitotic rate varied only 1.1%, experimentation was begun.

1, 3, 5, (10), 16-estratetraen-3-ol (#742), 17α methyl 1, 3, 5, (10), triene 3, 17 B diol (#837), and 9, 10, dimethyl 1, 2, benzanthracene (#788 or DMBA) were obtained in crystalline form from Dr. Max N. Huffman, Lasdon Foundation, Colorado Springs, Colorado (present address: Department of Biochemistry, Creighton University School of Medicine, Omaha, Nebraska). The experimental hydrocarbons were dissolved in filter-sterilized propylene glycol in concentrations ranging from 0.5 mg to 25.0 mg per milliliter.

The solutions were thoroughly mixed using a magnetic mixer and stirring bar. DMBA was difficult to dissolve and required long periods of mixing in order to dissolve it at a concentration of 20 mg per milliliter. It was necessary to prepare the hydrocarbons in high concentrations so as to keep the level of propylene glycol as low as possible in the experimental cultures. Appropriate amounts of the dissolved hydrocarbons were then dispensed into culture media with a tuberculin syringe and thoroughly mixed. At least ten identical replicate cultures were set up for each test concentration of hydrocarbon.

Coverslips with adherent cells were removed with forceps from both control and experimental cultures after 72 hours incubation at 21°C and placed in phosphate buffered saline for 20 minutes. They were then placed in Bouin's fixative for two minutes, followed by two changes of 80% ethanol (until yellow color disappeared), followed by two rinses of distilled deionized water. Cells were stained in Erhlich's hematoxylin (30 drops in 10 milliliters of water) for 15 minutes, then counterstained in 1% eosin for 1 minute. Coverslips were then rinsed in two changes of 95% ethanol and two changes of 100% ethanol. Cells were cleared in xylene and mounted over picolyte.

The mitotic indices were determined using the method of Paul (1960). Random counts of at least 1000 cells were made from the coverslip growth in each test culture using oil immersion. The mitotic index was calculated by dividing the total number of cells in mitosis by the total number of cells in mitosis by the total number of cells counted. This method afforded a total count of over 10,000 cells for each test concentration, and an overall mitotic index based on over 10,000 cells. Only cells in obvious stages of mitoses were counted. Fish cells, in comparison with mammalian L-fibroblasts, were more easily classified in the stages of prophase, and the instances of bizarre divisions were considerably lower.

The mitotic indices given here are lower than ones which would include the initial stages of prophase. The cells in initial prophase are

difficult to recognize with accuracy, especially from in vitro cells, and were therefore not counted. Counts were made using a dual key laboratory counter (Clay-Adams, Inc.), and all cytological examinations were made using a Bausch and Lomb binocular microscope.

Percentages of multinucleate cells were determined using similar procedures. All cells having two or more nuclei were classified as being multinucleate. This determination required a great deal of time in order to eliminate telophase cells and cells with lobulated nuclei. Only cells having obvious multiple interphase nuclei, and entirely separate nuclei were counted as multinucleate cells. All counts were made under oil immersion.

Each treatment group was compared with the control group and every other treatment group of the same hydrocarbon by calculating the least significant difference or lsd according to the method of Steel (1960). In each significant case, a significant F was calculated at the .05 level for each treatment group. In addition, Duncan's new multiple range test was computed. Correlation coefficients were calculated to test the possibility of correlation between rates of cell division and multinucleation.

CHAPTER IV

RESULTS

Mitotic Indices[1/]

742/FHM

Based on the total cell counts from all the cultures in the control group, FHM cultures had a mean mitotic index of 3.04% (2.7%-3.4%). Cell growth appeared as a well organized epithelial sheet. It was difficult to detect cell membranes in areas of contiguous growth. No cell clumps were present in any of the control cultures.

FHM cultures treated with 0.1 ppm #742 had an average mitotic index of 2.50% (1.32%-3.8%), a reduction over the controls. This slight inhibition was not significant at the .05 level, however it was significant at the .10 level. Nuclei of the treated cultures had much larger clumps of chromatin in comparison with the controls. There were no obvious morphological changes at this concentration.

Cell division in FHM cultures treated with 0.5 ppm #742 also was slightly inhibited with an average mitotic index of 2.3% (2.1%-2.5%). The inhibition was statistically significant at the .10 level but not at the .05 level. Cellular morphology was unchanged from the 0.1 ppm #742 treated cultures. No statistically significant different could be shown

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1/ See Tables I, IV, V, VI, VII, VIII and IX.

between the mitotic indices of the 0.1 and 0.5 ppm #742 treatment groups.

At 1.0 ppm #742, there was evidence of definite morphological effects. FHM cells grown at this concentration appeared more stellate and somewhat less epithelioid. The average mitotic index was 5.2% (4.5%-6.2%), a statistically significant increase at the .05 (but not at the .01) level over the controls.

Marked cytological changes were observed in FHM cultures treated with 5.0 ppm #742. Many cells were large and lightly stained with numerous vacuoles in the cytoplasm. Evidence of cytolysis was noted. Spaces were located between the cells and cells were not organized into a fully formed epithelial sheet. The average mitotic rate was 2.3% (1.09%-3.16%). Rates of cell division seemed to vary a great deal from one area of cell growth to another. This marked variation was not observed in the control cultures or in the cultures at lower concentrations. The average mitotic rate of the treated cultures was statistically lower than the controls at the .01 level.

At 10.0 ppm #742, the morphology of the treated cells was decidedly more fibroblast-like and normal cell division did not occur. No cells were observed in typical metaphase. Many hyperchromatic nuclei was calculated to be 6.2%. Many cells contained a normal appearing nucleus and a hyperchromatic mass in the cytoplasm. In some instances it appeared as if the mass were being extruded. Many cells were lysed.

Table I

SUMMARY OF EFFECTS OF # 742

Concentrations, ppm	Control	0.1	0.5	1.0	5.0	10.0
			Cell line FHM			
Mitotic Indices %	3.04	2.50	2.3	5.2	2.3	T
Range	(2.7-3.4)	(1.32-3.8)	(2.1-2.5)	(4.5-6.2)	(1.09-3.16)	
Significance level		.10	.10	.05	.01 *	
Multinucleation %	0.52	0.63	0.70	6.16	5.43	6.23
Range	(0.28-1.0)	(0.30-1.2)	(0.35-1.7)	(5.1-7.1)	(4.8-6.4)	(5.9-7.6)
Significance level		NS	NS	.001	.001	.001
			Cell line RTG-2			
Mitotic Indices %	3.3	3.1	3.0	2.09	2.52	4.11
Range	(2.9-4.5)	(2.8-4.4)	(2.5-4.5)	(1.77-2.67)	(1.35-2.44)	(3.52-4.94)
Significance level		NS	NS	.001	.001	.001
Multinucleation %	1.61	1.52	1.73	1.92	2.52	1.58
Range	(1.1-1.9)	(1.2-1.8)	(1.4-1.9)	(1.3-2.3)	(1.35-2.74)	(1.2-2.3)
Significance level		NS	NS	NS	.05	NS

NS = Not significant compared with control; T = toxic, accurate determination not possible;
* = compared with 1.0 ppm.

FHM cultures treated with 15.0 and 20.0 ppm #742 had only a few necrotic cells present.

742/RTG-2

The average mitotic index of the RTG-2 control cultures was found to be 3.30% (2.9%-4.5%). The cells were well-formed and typically fibroblastic. At concentrations of 0.1 and 0.5 ppm #742, only a slight decrease in the rate of cell division was observed in the RTG-2 treated cultures. However at 1.0 ppm #742, an average mitotic rate of 2.09% (1.77%-2.67%) was determined. This was found to be a statistically significant decrease at the .001 level. There were no observable effects on cellular morphology.

5.0 ppm #742 inhibited the rate of cell division of RTG-2 cultures to an average of 2.52% (1.35%-2.44%) statistically significant at .01 level. Slight morphological changes to a somewhat more rounded undifferentiated cell shape were observed. There were a few hyperchromatic nuclei present. Many normal appearing metaphase stages were seen. The cell sheet was less dense than in the control cultures.

At 10.0 ppm #742, the RTG-2 treated cultures had an average mitotic rate of 4.11% (3.52%-4.94%), an increase statistically significant at .001 level. All stages of mitosis were observed, however some of the chromosomes appeared dense and thickened. Prophase nuclei contained prominent, well-spaced chromatin networks. A few hyperchomatic nuclei were seen.

837/FHM

The mitotic indices of FHM cultures treated with 0.1 and 0.5 ppm #837 did not differ statistically from the control cultures; however cultures treated with 1.0 ppm #837 had an average mitotic index of 1.68% (1.1%-2.4%) as compared with 3.04% in the control group. This reduction was statistically significant at the .001 level. Cells did not appear abnormal in this treatment group.

At 5.0 ppm #837 strongly inhibited cell division in the FHM cultures, the average rate of cell division was 0.65% (0.3%-1.0%). This was a statistically significant decrease at the .001 level from 1.0 ppm cultures. The cellular membranes at this concentration were indistinct and the cells appeared to be coalescing. Nuclei were very lightly stained. Karyorrhexis was observed.

The 10.0 ppm #837 completely inhibited FHM mitosis. The nuclei were very indistinct. Fewer necrotic cells were noted in this treatment group in contrast to the number produced by the same concentration of #742. Many of the nuclei in the 10.0 ppm #837 treatment group were lobulated. The cellular arrangement was "tile-like" in that even spaces were present between the cells. This probably indicated the lack of cellular division or movement since attachment to the glass.

FHM cultures treated with 20.0 and 40.0 ppm #837 also had little evidence of mitotic activity. Nuclei of the cells were very pale and hardly distinguishable from the cytoplasm except for the peripheral chromatin located adjacent to the nuclear envelope.

Table II

SUMMARY OF EFFECTS OF #837

Concentrations ppm	Control	0.1	0.3	0.5	1.0	5.0	10.0
			Cell line FHM				
Mitotic Indices %	3.04	3.05		2.87	1.68	0.65	0
Range	(2.7-3.4)	(2.8-3.7)		(2.1-3.3)	(1.1-2.4)	(0.3-1.0)	
Significance level		NS		NS	.001	.001	
Multinucleation %	0.52	0.69		0.76	5.12	8.69	11.66
Range	(0.28-1.0)	(0.32-1.1)		(0.29-1.4)	(3.96-6.87)	(7.0-10.7)	(7.7-18.7)
Significance level		NS		NS	.001	.001	.001
			Cell line RTG-2				
Mitotic Indices %	3.3	3.10	11.29	12.36	T		
Range	(2.9-4.5)	(2.6-3.68)	(8.41-14.49)	(10.56-14.59)			
Significance level		.05	.001	.001			
Multinucleation %	1.61	3.21	2.15	25.69	T		
Range	(1.1-1.9)	(2.7-3.7)	(1.6-2.7)	(19.6-31.7)			
Significance level		.001	.001	.001			

NS = Not significant compared with control.

T = Toxic, accurate determination not possible.

Some cells were present in cultures treated with 60.0 and 80.0 ppm #837, however they were extremely pale and necrotic. No cells were present in cultures treated with 100.0 ppm #837.

#837/RTG-2

RTG-2 cultures treated with #837 exhibited effects at much lower concentrations than did FHM cells. At 0.1 ppm #837, RTG-2 cells had an average mitotic rate of 3.10% (2.60%-3.68%), only slightly lower than the control index of 3.3%. This decrease was found statistically significant at the .05 level, but not at the .01 level when compared with the controls.

The 0.3 ppm #837 treated RTG-2 cultures had an average mitotic index of 11.29% (8.41%-14.49%), a considerable increase over the control cultures (3.3%) and statistically significant at the .001 level. The 0.5 ppm #837 cultures had an average mitotic index of 12.36% (10.56%-14.59%). This increase was not statistically significant over the 0.3 ppm #837 treatment group, but was statistically significant over the control group at the .001 level. Cells in these two treatment groups contained well-stained nuclei, in comparison with the FHM treated cells; the cellular morphology was anaplastic and large numbers of giant cells were seen.

At 1.0 ppm #837, only half of the cultures contained any cell growth. These cells had smaller nuclei and reduction in the amount of cytoplasm. No metaphase spindles were observed. Some cells appeared to have lost their nuclear envelope and chromatin was clumped into discrete

masses giving the appearance of a normoblast cell before nuclear extrusion. No giant cells were seen. Only three RTG-2 cultures out of ten in the 5.0 ppm #837 treatment group contained any cells. These cells exhibited the same but exaggerated cytological appearance as the 1.0 ppm #837 cultures. The percentage of "normoblast-like" cells was considerably higher, approximately 44.0%. Evidence of karyorrhexis was observed. No metaphase spindles were seen.

At 10.0 and 20.0 ppm #837, the same conditions were noted, but even fewer cells were present. These cells were much larger, and no longer recognizable as fibroblasts. The 40.0 ppm #837 was lethal to RTG-2 cells.

DMBA/FHM

FHM cultures treated with 0.1 and 0.5 ppm DMBA did not have mitotic indices statistically different from the controls. At 1.0 ppm DMBA, FHM treated cultures had an average mitotic index of 2.32% (1.2%-3.34%). This was slightly lower, but not a statistically significant reduction as compared with controls. Mild cytotoxicity was noted. At 5.0 ppm and 10.0 ppm DMBA, mitosis in the FHM cultures was stopped. Many cells in these cultures were extremely abnormal with a reduction in cytoplasm, clumped chromatin and basophilic cytoplasm. The chromatin often formed one or more large clumps in the nucleus, and the remainder became evenly distributed on the inner surface of the nuclear membrane. Many hyperchromatic cells had multiple cytoplasmic constrictions giving the impression that cytokinesis was occurring

Table III

SUMMARY OF DMBA EFFECTS

Concentration ppm	Control	0.1	0.5	1.0	5.0	10.0	20.0
			Cell line FHM				
Mitotic Indices %	3.04	3.2	2.91	2.32	0	0	0
Range	(2.7-3.4)	(2.6-3.5)	(2.3-3.5)	(1.2-3.34)			
Significance level		NS	NS	NS			
Multinucleation %	0.52	0.92	5.53	6.69	6.1	1.56	1.23
Range	(0.28-1.0)	(0.4-1.3)	(4.20-7.12)	(5.30-8.58)	(5.0-8.7)		(1.0-1.57)
Significance level		NS	.001	.001	.001	.001	.001
			Cell line RTG-2				
Mitotic Indices %	3.3	2.9	0.96	0.4	0	0	0
Range	(2.9-4.5)	(2.5-3.2)	(0.49-1.69)	(0.19-1.19)			
Significance level		NS	.001	.001			
Multinucleation %	1.61	1.57	1.15	3.3	T		
Range	(1.1-1.9)	(1.0-2.0)	(0.5-1.9)	(2.2-4.2)			
Significance level		NS	.01	.001			

NS = Not significant compared with control.
T = Toxic, accurate determination not possible.

without karyokinesis.

The percentage of hyperchromatic FHM cells was calculated to be 67.9% at 10.0 ppm DMBA. Cytoplasmic vaculation was observed to some extent in nearly all of the cells.

Identical cytotoxic effects were noted in the 20.0 ppm DMBA FHM cultures with an increase in the percentage of affected cells to 91.4%. This figures represents a statistically (.01 level) significant increase in number of affected cells over the 10.0 ppm DMBA treated cultures.

At 40.0 ppm DMBA treated FHM cultures contained a few very small, hyperchromatic spindle shaped cells. No cells were present in cultures treated with 45.0 ppm DMBA.

DMBA/RTG-2

A slight, but statistically insignificant, inhibition was observed in RTG-2 cultures treated with 0.1 ppm DMBA, however RTG-2 cultures were strongly inhibited at 0.5 ppm DMBA. The average mitotic index at this concentration was 0.96% (0.49%-1.69%) as compared with 3.3% in the controls. This reduction was statistically significant at the .001 level. Cells appeared normal with no evidence of cytotoxic effects.

The 1.0 ppm DMBA cultures had an average mitotic rate of only 0.4%(0.19%-1.19%) which was a stististically significant reduction over the 0.5 ppm treated cultures. No cytotoxic effects were observed.

At concentrations of 5.0, 10.0 and 20.0 ppm DMBA, no mitotic activity was observed in any of the treated cultures. Cells were hyperchromatic with vaculated cytoplasm, and highly necrotic. No cells

were present in cultures treated with 40.0 ppm DMBA.

Multinucleation[2/]

#742/FHM

Control FHM cultures has an average percentage of multinucleation of 0.52% (0.25%-1.0%). FHM cultures treated with 0.1 ppm and 0.5 ppm #742 had percentages of multinucleation which deviated only slightly from the controls. FHM cultures treated with 1.0 ppm #742 had an average percentage of multinucleation of 6.16% (5.1%-7.1%), a statistically significant increase at the .001 level.

Percentage of multinucleation in the 5.0 ppm #742 cultures was 5.43% (4.8%-6.4%), a reduction of 0.73% from the 1.0 ppm #742 cultures. The 10.0 ppm #742 FHM cultures had an average of 6.23% (5.5%-7.2%). No statistically significant differences were found among the 1.0, 5.0, and 10.0 ppm #742 FHM cultures.

#742/RTG-2

Control RTG-2 cultures had an average percentage of multinucleation of 1.61% (1.1%-1.9%). RTG-2 cultures treated with 0.1, 0.5 and 1.0 ppm #742 did not differ statistically from the control percentage of multinucleation. At 5.0 ppm #742 treated RTG-2 cultures had an average percentage of 2.52% (1.35%-2.74%). This increase was statistically significant at the .05 level, but not at the .01 level. At 10.0 ppm #742, RTG-2 cultures had an average multinucleation of 1.58% (1.2%-2.3%) which was not statistically significant compared

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2/ See Tables I, X, XI, XII and XIII.

with the controls.

#837/FHM[3/]

Statistically significant differences in percentage multinucleation was not found at 0.1 or 0.5 ppm #837, however 1.0 ppm #837 treated FHM cultures had an average percentage of multinucleation of 5.12% (3.96%-6.87%) as compared with 0.52% in the controls. This increase was found statistically significant at the .001 level. At 5.0 ppm #837, FHM cultures had an average of 8.69% (7.0%-10.70%) multinucleated cells. This percentage was statistically significant as compared with the controls. Compared statistically with 1.0 ppm #837 FHM cultures, 5.0 ppm #837 significantly increased the percentage of multinucleation at the .001 level.

The average percentage of multinucleation was 11.66% (7.70%-18.70%) in the 10.0 ppm #837 treated FHM cultures. This increased percentage was significant at the .05 level, when compared with the 5.0 ppm #837 cultures and significant at the .001 level compared with controls.

At 20.0 ppm FHM cultures had an average percentage multinucleation of 11.46% (10.50%-12.90%). This figure did not differ statistically from the 10.0 ppm #837 treated FHM cultures. The 40.0 ppm #837 had an average percentage of multinucleation of 12.27% (8.50%-13.35%). This was not statistically different from the 20.0 ppm #837 FHM cultures.

[3/] See Tables II, XIV and XV.

#837/RTG-2[4/]

As was observed with the mitotic indices, multinucleation effects produced by #837 were noted at much lower concentrations with the RTG-2 cell line than with the FHM. At 0.1 ppm #837, RTG-2 cultures had an average percentage multinucleation of 3.21%(2.70%-3.70%) as compared with 1.61% (1.10%-1.90%) in the controls. This was a significant increase (.001) over the controls. At 0.3 ppm #837, RTG-2 cultures had an average percentage multinucleation of 2.15% (1.60%-2.70%) which was also a significant increase over the controls. As compared with the 0.1 ppm cultures, 0.3 ppm had a significantly lower percentage multinucleation at the .001 level.

At 0.5 ppm #837 strongly increased the percentage multinucleation from that of the 0.3 ppm #837 cultures to an average of 25.69% (19.60%-31.70%), significant at the .001 level. At concentrations of 1.0, 5.0 and 10.0 ppm #837, it was impossible to determine the percentages of multinucleate cells due to cytotoxic effects, but as far as could be determined, the percentage was quite low.

DMBA /FHM[5/]

FHM cultures treated with 0.1 ppm DMBA did not have a statistically significant increase in the percentage of multinucleate cells. At 0.5 ppm DMBA, the average percentage multinucleation was 5.53% (4.20%-7.12%) and at 1.0 ppm DMBA, the average was 6.69% (5.30%-8.50%). These increases were found statistically significant at the .001

4/ See Tables II, XVI, XVII and XVIII. 5/ See Table III.

level when compared to the controls. At 5.0 ppm DMBA, the percentage was 6.1% (5.0%-8.7%) which was not a statistically significant decrease from the 1.0 ppm cultures. At 10.0 ppm DMBA, the percentage decreased to an average of 1.56% (1.0%-2.3%) which was a significant decrease as compared with the 1.0 ppm DMBA cultures at the .001 level. However, when compared statistically with the control percentage, multinucleation in the 10.0 ppm DMBA cultures was significantly higher at the .001 level.

At 20.0 ppm DMBA, the average percentage multinucleation was 1.23% (1.0%-1.57%), a slight decrease compared with the 10.0 ppm cultures which was significant at the .01 level, but not at the .001 level.

DMBA/RTG-2[6/]

No statistically significant changes were noted in the percentage of multinucleate cells in the 0.1 ppm DMBA treated RTG-2 cultures, however at 0.5 ppm DMBA, RTG-2 cultures had an average percentage multinucleation of 1.15%(0.50%-1.90%) as compared with the average control percentage of 1.61%. This decrease was significant at the .01 level but not at the .001 level. The 1.0 ppm DMBA caused an increase in the percentage of multinucleation of RTG-2 cultures to an average of 3.30% (2.2%-4.2%), a statistically significant increase over the 0.5 ppm DMBA cultures at the .001 level.

At 5.0 and 10.0 ppm DMBA, RTG-2 cells showed marked toxic effects and the percentage of multinucleation could not be accurately determined.

[6/] See Tables III, XIX and XX.

Table IV

MITOTIC INDEX CONTROL CULTURES FHM

Culture Tube No.	1	2	3	4	5	6	7	8	9	10	TOTALS
Total Cells	1116	1154	1382	1482	1205	1118	1464	1167	1384	1090	12,562
Total Mitotic	44	40	39	41	35	32	45	37	39	30	382
Mitotic Index	3.90%	3.4%	2.80%	2.7%	2.95%	2.87%	3.1%	3.1%	2.8%	2.7%	3.04%

Table V

MITOTIC INDEX, 0.1 ppm #742/FHM

Culture Tube No.	1	2	3	4	5	6	7	8	9	10	TOTALS
Total Cells	1132	1077	1065	1165	1068	1058	1127	1156	1027	1025	10,900
Total Mitotic	15	27	15	27	41	37	33	31	27	26	279
Mitotic Index	1.32%	2.50%	1.40%	2.3%	3.8%	3.4%	2.9%	2.6%	2.6%	2.5%	2.5%

Table VI

MITOTIC INDEX, 0.5 ppm #742/FHM

Culture Tube No.	1	2	3	4	5	6	7	8	9	10	TOTALS
Total Cells	1053	1044	1046	1058	1010	1050	1060	1090	1045	1052	10,508
Total Mitotic	26	25	25	24	22	25	27	28	24	26	252
Mitotic Index	2.4%	2.3%	2.3%	2.2%	2.1%	2.3%	2.5%	2.5%	2.2%	2.4%	2.3%

Table VII

MITOTIC INDICES, 1.0 ppm #742/FHM

Culture Tube No.	1	2	3	4	5	6	7	8	9	10	TOTALS
Total Cells	1152	1233	1325	1067	1130	1166	1142	1076	1038	1157	10,344
Total Mitotic	55	72	77	53	62	73	69	47	50	59	548
Mitotic Indices	4.5%	5.8%	5.8%	4.96%	5.48%	6.26%	6.0%	4.3%	4.8%	5.0%	5.2%

Table VIII

MITOTIC INDICES, 5.0 ppm #742/FHM

Culture Tube No.	1	2	3	4	5	6	7	8	9	10	TOTALS
Total Cells	1063	1233	1101	1114	1018	1109	1074	1093	1197	1256	11,258
Total Mitotic	34	39	31	30	19	31	28	12	22	23	269
Mitotic Indices	3.1%	3.16%	2.8%	2.6%	1.86%	1.8%	2.6%	1.09%	1.8%	1.83%	2.3%

Table IX

MITOTIC INDICES, 10.0 ppm #742/FHM

Culture Tube No.	1	2	3	4	5	6	7	8	9	10	TOTALS
Total Cells	1016	1020	1019	1003	1027	1104	1080	1168	1103	923	10,463
Mitotic Cells	78	65	59	52	82	62	65	72	68	55	658
Mitotic Indices	7.6%	6.3%	5.7%	5.1%	7.9%	5.6%	6.0%	6.1%	6.1%	5.9%	6.2%

Table X

MITOTIC INDICES, CONTROLS/RTG-2

Culture Tube No.	1	2	3	4	5	6	7	8	9	10	TOTALS
Total Cells	1000	1001	1050	1014	1002	1010	1014	1010	1018	1020	10, 139
Total Mitotic	38	46	32	35	41	43	36	33	30	32	336
Mitotic Indices	3.8%	4.59%	3.5%	3.45%	4.0%	4.25%	3.55%	3.2%	2.9%	3.1%	3.3%

Table XI

MITOTIC INDICES, 1.0 ppm #742/RTG-2

Culture Tube No.	1	2	3	4	5	6	7	8	9	10	TOTALS
Total Cells	1059	1008	1026	1012	1040	1000	1001	1003	1004	1003	10, 156
Total Mitotic	25	27	20	18	21	18	23	20	22	20	214
Mitotic Indices	2.36%	2.67%	1.94%	1.77%	2.0%	1.8%	2.29%	1.99%	2.1%	2.0%	2.10%

Table XII

MITOTIC INDICES, 5.0 ppm #742/RTG-2

Culture Tube No.	1	2	3	4	5	6	7	8	9	10	TOTALS
Total Cells	1018	1010	1081	1040	1072	1031	1000	1055	1013	1033	10, 353
Total Mitotic	27	27	28	27	29	14	26	29	26	28	261
Mitotic Indices	2.65%	2.67%	2.59%	2.59%	2.70%	1.35%	2.60%	2.74%	2.56%	2.71%	2.52%

Table XIII

MITOTIC INDICES, 10.0 ppm #742/RTG-2

Culture Tube No.	1	2	3	4	5	6	7	8	9	10	TOTALS
Total Cells	1031	1043	1055	1061	1015	1061	1207	1023	1025	1030	10,551
Total Mitotic	51	41	39	42	38	46	51	36	40	50	434
Mitotic Indices	4.94%	3.93%	3.69%	3.95%	3.74%	4.34%	4.22%	3.52%	3.90%	4.85%	4.11%

Table XIV

MITOTIC INDICES, 1.0 ppm #837/FHM

Culture Tube No.	1	2	3	4	5	6	7	8	9	10	TOTALS
Total Cells	1097	1068	1022	1015	1020	1000	1090	1014	1010	1019	10,355
Mitotic Cells	27	14	16	25	11	14	20	24	15	12	178
Mitotic Indices	2.4%	1.3%	1.5%	2.4%	1.07%	1.4%	1.8%	2.3%	1.48%	1.1%	1.7%

Table XV

MITOTIC INDICES, 5.0 ppm #837/FHM

Culture Tube No.	1	2	3	4	5	6	7	8	9	10	TOTALS
Total Cells	1011	1020	1030	1008	1093	1106	1020	1080	1019	1007	10,394
Mitotic Cells	11	7	10	12	4	4	9	5	8	5	75
Mitotic Indices	1.0%	0.6%	0.9%	1.1%	0.3%	0.3%	0.8%	0.4%	0.7%	0.4%	0.7%

Table XVI

MITOTIC INDICES, 0.1 ppm #837/RTG-2

Culture Tube No.	1	2	3	4	5	6	7	8	9	10	TOTALS
Total Cells	1030	1197	1050	1020	1113	1001	1036	1044	1025	1010	10,526
Mitotic Cells	37	31	35	27	41	30	32	36	29	29	327
Mitotic Indices	3.59%	3.2%	3.33%	2.64%	3.68%	2.99%	3.08%	3.44%	2.82%	2.87%	3.0%

Table XVII

MITOTIC INDICES, 0.3 ppm #837/RTG-2

Culture Tube No.	1	2	3	4	5	6	7	8	9	10	TOTALS
Total Cells	1053	1092	1054	1066	1014	1040	1022	1032	1050	1012	10,435
Mitotic Cells	122	116	121	125	147	117	86	120	125	99	1,178
Mitotic Indices	11.58%	11.62%	11.48%	11.72%	14.49%	11.25%	8.41%	11.62%	11.90%	9.78%	11.28%

Table XVIII

MITOTIC INDICES, 0.5 ppm #837/RTG-2

Culture Tube No.	1	2	3	4	5	6	7	8	9	10	TOTALS
Total Cells	1005	1007	1030	1025	1012	1010	1039	1008	1027	1022	10,185
Mitotic Cells	107	147	109	139	133	140	147	117	109	113	1,261
Mitotic Indices	10.65%	14.59%	10.58%	13.56%	13.14%	13.68%	14.14%	11.60%	10.61%	11.05%	12.38%

Table XIX

MITOTIC INDICES, 0.5 ppm DMBA/RTG-2

Culture Tube No.	1	2	3	4	5	6	7	8	9	10	TOTALS
Total Cells	1005	1021	1010	1020	1015	1010	1019	1000	1015	1020	10,135
Mitotic Cells	17	16	6	5	12	10	5	8	9	9	97
Mitotic Indices	1.69%	1.56%	0.59%	0.49%	1.1%	0.99%	0.49%	0.8%	0.88%	0.88%	0.96%

Table XX

MITOTIC INDICES, 1.0 ppm DMBA/RTG-2

Culture Tube No.	1	2	3	4	5	6	7	8	9	10	TOTALS
Total Cells	1006	1010	1008	1002	1006	1008	1002	1010	1006	1009	10,067
Mitotic Cells	2	6	7	12	5	6	5	5	6	4	58
Mitotic Indices	0.19%	0.59%	0.69%	1.19%	0.49%	0.59%	0.49%	0.49%	0.59%	0.39%	0.4%

Table XXI

MITOTIC INDICES, 1.0 ppm DMBA/FHM

Culture Tube No.	1	2	3	4	5	6	7	8	9	10	TOTALS
Total Cells	1118	1064	1142	1029	1080	1054	1017	1046	1053	1131	10,734
Mitotic Cells	19	13	32	16	32	35	20	24	30	29	250
Mitotic Indices	1.66%	1.20%	2.80%	1.59%	2.96%	3.34%	1.96%	2.29%	2.84%	2.56%	2.32%

Table XXII

PERCENTAGE MULTINUCLEATION, CONTROL FHM

Culture Tube No.	1	2	3	4	5	6	7	8	9	10	TOTALS
Total Cells	1075	1063	1023	1076	1052	1042	1037	1023	1087	1008	10,486
Multinucleate	9	4	3	5	4	3	4	4	10	8	54
Multinucleation	0.85%	0.40%	0.30%	0.5%	0.35%	0.28%	0.35%	0.38%	1.0%	0.78%	0.52%

Table XXIII

PERCENTAGE MULTINUCLEATION, 1.0 ppm #742/FHM

Culture Tube No.	1	2	3	4	5	6	7	8	9	10	TOTALS
Total Cells	1014	1053	1023	1018	1007	1029	1062	1037	1031	1022	10,296
Multinucleate	73	60	56	71	67	55	54	71	61	67	635
Multinucleation	7.1%	5.7%	5.5%	7.0%	6.7%	5.3%	5.1%	6.8%	5.9%	6.5%	6.1%

Table XXIV

PERCENTAGE MULTINUCLEATION, 5.0 ppm #742/FHM

Culture Tube No.	1	2	3	4	5	6	7	8	9	10	TOTALS
Total Cells	1021	1026	1034	1059	1042	1006	1009	1051	1046	1072	10,366
Multinucleate	55	59	52	59	53	49	48	67	61	58	561
Multinucleation	5.4%	5.8%	5.0%	5.6%	5.1%	4.9%	4.8%	6.4%	5.9%	5.4%	5.4%

Table XXV

PERCENTAGE MULTINUCLEATION, 10.0 ppm #742/FHM

Culture Tube No.	1	2	3	4	5	6	7	8	9	10	TOTALS
Total Cells	1023	1056	1011	1079	1009	1103	1062	1058	1041	1027	10,469
Multinucleate	58	67	67	69	59	69	76	58	72	62	657
Multinucleation	5.7%	6.4%	6.7%	6.4%	5.9%	6.3%	7.2%	5.5%	6.9%	6.0%	6.2%

Table XXVI

PERCENTAGE MULTINUCLEATION, RTG-2 CONTROLS

Culture Tube No.	1	2	3	4	5	6	7	8	9	10	TOTALS
Total Cells	1143	1158	1036	1095	1042	1007	1213	1012	1105	1187	10,798
Multinucleate	20	20	19	19	11	12	18	19	22	18	178
Multinucleation	1.74%	1.76%	1.9%	1.8%	1.1%	1.2%	1.4%	1.8%	1.9%	1.5%	1.6%

Table XXVII

PERCENTAGE MULTINUCLEATION, 1.0 ppm #742/RTG-2

Culture Tube No.	1	2	3	4	5	6	7	8	9	10	TOTALS
Total Cells	1142	1076	1081	1024	1039	1022	1063	1097	1105	1121	10,770
Multinucleate	20	17	13	10	16	14	19	20	20	16	175
Multinucleation	1.8%	1.6%	1.2%	2.0%	1.5%	1.4%	1.8%	1.9%	1.8%	1.4%	1.6%

Table XXVIII

PERCENTAGE MULTINUCLEATION, 5.0 ppm #742/RTG-2

Culture Tube No.	1	2	3	4	5	6	7	8	9	10	TOTALS
Total Cells	1018	1010	1081	1040	1072	1031	1000	1055	1033	1013	10,353
Multinucleate	15	17	13	19	20	11	17	16	18	13	159
Multinucleation	1.5%	1.7%	1.2%	1.8%	1.9%	1.1%	1.7%	1.5%	1.8%	1.3%	1.5%

Table XXIX

PERCENTAGE MULTINUCLEATION, 10.0 ppm #742/RTG-2

Culture Tube No.	1	2	3	4	5	6	7	8	9	10	TOTALS
Total Cells	1048	1082	1031	1025	1031	1055	1167	1104	1069	1044	10,656
Multinucleate	14	15	24	13	17	16	16	18	20	13	166
Multinucleation	1.4%	1.7%	2.3%	1.3%	1.6%	1.5%	1.4%	1.6%	1.8%	1.2%	1.5%

Table XXX

PERCENTAGE MULTINUCLEATION, 1.0 ppm #837/FHM

Culture Tube No.	1	2	3	4	5	6	7	8	9	10	TOTALS
Total Cells	1021	1018	1052	1073	1008	1048	1037	1031	1025	1082	10,395
Multinucleate	42	70	54	62	67	42	63	43	46	43	532
Multinucleation	4.13%	6.87%	5.13%	5.77%	6.64%	4.0%	6.07%	4.17%	4.48%	3.97%	5.1%

Table XXXI

PERCENTAGE MULTINUCLEATION, 5.0 ppm #837/FHM

Culture Tube No.	1	2	3	4	5	6	7	8	9	10	TOTALS
Total Cells	1005	1032	1062	1047	1039	1003	1013	1029	1037	1042	40, 309
Multinucleate	82	95	88	99	95	73	87	97	73	112	901
Multinucleation	8.15%	9.2%	8.28%	9.5%	9.1%	7.2%	8.5%	9.4%	7.0%	10.7%	8.7%

Table XXXII

PERCENTAGE MULTINUCLEATION, 10.0 ppm #837/FHM

Culture Tube No.	1	2	3	4	5	6	7	8	9	10	TOTALS
Total Cells	1000	1009	1024	1009	1034	1017	1028	1023	1011	1021	10, 176
Multinucleate	187	134	79	90	137	92	128	116	87	136	1, 186
Multinucleation	18.7%	13.28%	7.7%	8.9%	13.3%	9.04%	12.45%	11.3%	8.6%	13.3%	11.6%

Table XXXIII

PERCENTAGE MULTINUCLEATION, 20.0 ppm #837/FHM

Culture Tube No.	1	2	3	4	5	6	7	8	9	10	TOTALS
Total Cells	1010	1009	1000	1026	1008	1006	1020	1025	1003	1014	10, 124
Multinucleate	120	121	126	123	130	105	97	105	123	129	1, 179
Multinucleation	11.88%	11.99%	12.6%	12.0%	12.9%	10.5%	9.5%	10.24%	12.16%	12.77%	11.6%

Table XXXIV

PERCENTAGE MULTINUCLEATION, 40.0 ppm #837/FHM

Culture Tube No.	1	2	3	4	5	6	7	8	9	10	TOTALS
Total Cells	1017	1006	1031	1028	1025	1010	1023	1019	1015	1011	10,185
Multinucleate	133	110	124	123	166	86	121	127	135	124	1,249
Multinucleation	13.07%	10.9%	12.02%	11.96%	16.19%	8.5%	11.9%	12.47%	13.35%	12.3%	12.2%

Table XXXV

PERCENTAGE MULTINUCLEATION, 0.1 ppm #837/RTG-2

Culture Tube No.	1	2	3	4	5	6	7	8	9	10	TOTALS
Total Cells	1005	1023	1154	1001	1082	1040	1302	1121	1029	1061	10,818
Multinucleate	32	33	43	32	30	32	46	38	31	34	351
Multinucleation	3.2%	3.3%	3.7%	3.1%	2.7%	3.0%	3.5%	3.4%	3.0%	3.2%	3.24%

Table XXXVI

PERCENTAGE MULTINUCLEATION, 0.3 ppm #837/RTG-2

Culture Tube No.	1	2	3	4	5	6	7	8	9	10	TOTALS
Total Cells	1032	1073	1091	1051	1008	1018	1070	1080	1050	1008	10,481
Multinucleate	24	26	20	18	22	23	25	18	24	28	228
Multinucleation	2.32%	2.42%	1.83%	1.71%	2.18%	2.2%	2.3%	1.6%	2.2%	2.7%	2.17%

Table XXXVII

PERCENTAGE MULTINUCLEATION, 0.5 ppm #837/RTG-2

Culture Tube No.	1	2	3	4	5	6	7	8	9	10	TOTALS
Total Cells	1085	1006	1107	1194	1009	1025	1038	1074	1062	1043	10,643
Multinucleate	224	258	290	354	196	324	209	287	311	282	2,739
Multinucleation	20.61%	25.7%	26.2%	30.0%	19.6%	31.7%	20.1%	26.7%	29.3%	27.0%	25.7%

Table XXXVIII

PERCENTAGE MULTINUCLEATION, 1.0 ppm DMBA/FHM

Culture Tube No.	1	2	3	4	5	6	7	8	9	10	TOTALS
Total Cells	1056	1033	1047	1007	1079	1057	1029	1036	1077	1092	10,513
Multinucleate	42	82	87	76	92	69	55	61	81	63	732
Multinucleation	6.2%	7.9%	8.3%	7.7%	8.5%	6.5%	5.3%	5.9%	7.5%	5.8%	6.9%

Table XXXIX

PERCENTAGE MULTINUCLEATION, 10.0 ppm DMBA/FHM

Culture Tube No.	1	2	3	4	5	6	7	8	9	10	TOTALS
Total Cells	1036	1176	1114	1169	1021	1017	1056	1047	1043	1006	10,695
Multinucleate	24	18	12	14	16	10	20	19	14	19	121
Multinucleation	2.3%	1.5%	1.1%	1.2%	1.6%	1.0%	1.9%	1.8%	1.3%	1.9%	1.1%

Table XL

PERCENTAGE MULTINUCLEATION, 20.0 ppm DMBA/FHM

Culture Tube No.	1	2	3	4	5	6	7	8	9	10	TOTALS
Total Cells	1081	1003	1101	1075	1033	1091	1071	1095	1105	1078	10, 733
Multinucleate	12	16	9	18	11	14	10	12	14	15	131
Multinucleation	1.1%	1.57%	0.9%	1.7%	1.01%	1.3%	1.0%	1.1%	1.26%	1.4%	1.2%

Table XLI

PERCENTAGE MULTINUCLEATION, 0.5 ppm DMBA/RTG-2

Culture Tube No.	1	2	3	4	5	6	7	8	9	10	TOTALS
Total Cells	1005	1025	1002	1056	1045	1081	1109	1069	1077	1027	10, 496
Multinucleate	19	13	6	9	14	11	16	15	8	11	122
Multinucleation	1.9%	1.2%	0.5%	0.9%	1.3%	1.0%	1.5%	1.4%	0.7%	1.1%	1.1%

Table XLII

PERCENTAGE MULTINUCLEATION, 1.0 ppm DMBA/RTG-2

Culture Tube No.	1	2	3	4	5	6	7	8	9	10	TOTALS
Total Cells	1006	1010	1008	1002	1006	1008	1002	1010	1006	1009	10, 067
Multinucleate	32	40	81	36	36	42	25	22	22	36	372
Multinucleation	3.2%	4.0%	8.1%	3.6%	3.5%	4.2%	2.5%	2.2%	2.2%	3.6%	3.6%

Table XLIII

DIVISION RATE SIGNIFICANCE

Chemical	Cell Line	Chemical Levels (ppm)*
#742	FHM	5.0 0.5 0.1 Control 1.0 10.0
#742	RTG-2	1.0 5.0 Control 10.0
#788	FHM	1.0 Control (Significance exists at .02 level)
#788	RTG-2	1.0 0.5 Control
#837	FHM	10.0 5.0 1.0 Control
#837	RTG-2	0.1 Control 0.3 0.5
		MULTINUCLEATION SIGNIFICANCE
#742	FHM	Control 5.0 1.0 10.0
#742	RTG-2	10.0 Control 1.0 5.0
#837	FHM	5.0 1.0 Control 10.0 20.0 40.0
#837	RTG-2	Control 0.3 0.1 0.5
#788	FHM	Control 20.0 10.0 1.0
#788	RTG-2	0.5 Control 1.0

* Any two means not underscored by the same line are significantly different. Any two means underscored by the same line are not significantly different.

CHAPTER V

DISCUSSION

There are a vast number of possible actions that chemical agents might have in altering cell division, inducing hyperplasia, evoking malignant transformation, or modifying morphology. Many theoretical explanations of the manner in which chemicals might alter cellular activity have been proposed; and the data herein shed little light on these crucial problems.

As regards inhibition of mitosis, one must consider at least three major events which occur if a cell is to divide; chromosomal reproduction, chromosomal movement and cytoplasmic cleavage. Blockage of any one of these events results in termination of cell division at that point and failure of later events to occur (Gelfant, 1963).

Inhibition at prophase will occur if the nucleolous is damaged (Gaulden and Perry, 1958; Gaulden, 1960), or if the nuclear membrane breakdown is prevented (Hadder and Wilson, 1958). Eigsti and Dustin (1955) reported that anaphase will not occur if the spindle is disoriented during metaphase. If anaphase movement of chromosomes is inhibited, telophase will not occur (Ris, 1949). The final act of cell division, that of cytoplasmic cleavage, must also occur if two normal uninucleate cells

are produced, and this too can be chemically blocked (Morsland, 1956; Prescott, 1961).

In order for the cycle to repeat itself, chromosomes must uncoil from the condensed state following telophase; failure to do so results in telophase blockage (Bucher and Mazia, 1960). Each of these events entails complicated smaller steps in the overall event. For example, Kanazier and Errera (1954) inhibited DNA synthesis in Escherichia coli but were able to demonstrate the accumulation of DNA precursors although DNA synthesis itself was inhibited.

Mazia (1961) proposed the concept of "points of no return" which views the progress of mitotic events as "unblockable" once the event is induced; for example, once DNA synthesis is stimulated to occur, DNA blocking agents will not be effective.

The mitotic cycle has long been characterized only in its most obvious phases with little regard for interphase. The interphase stage has in more recent years been studied in detail and characterized in vivo and in vitro (Gelfant, 1962). One must take these stages into consideration in order to find a point at which a chemical might initiate or inhibit activity, particularly with reference to DNA synthesis or other non-observable phenomena. The interphase stages have been termed Gap_1 (G_1), Synthesis of DNA (S) and Gap_2 (G_2). Inhibition at G_1, that period immediately following cell division, has been described by Bollum (1960) and Lajtha (1958), S blockage of DNA and G_2 blockage, that period just

before prophase, was described by Painter and Robertson (1959). If a cell is inhibited at any one of these interphase stages, it will not progress beyond that point (Biesele, 1958).

Mitotic inhibition, as opposed to mitotic stimulation, could occur at any single point in the complex cyclic chain of events. Mitotic stimulation, however, is difficult to interpret in these terms, except as a regulator or some part of the regulatory mechanism of the overall process which may be influenced. Also one must consider, in this same context, growth (as a consequence of cell division and increased protoplasm), normal cell division for replacement and repair, hyperplasia and malignant transformation.

Especially needed in considering the present data is a theory which will explain to some extent the differentiation observed in cell cultures. The author feels this is best explained by the theory of Pardee (1964) which in its simplest terms holds the control of cell division and cellular differentiation to be under regulatory molecules from the environment, and failure of these substances to enter the cell, or their failure to act once inside the cell, results in loss of control and an increase in the rate of cell division. The theory has support from the findings that molecules of surprisingly large sized can pass from the environment to a cell. Berrill (1943) put forth long ago the concept that carcinogens were cell-surface-active compounds. More recently, Willmer (1961) reported evidence that even hormones can modify the permeability of

the cell membranes to various metabolites, which may in part explain some of the actions of #742 and #837 whose steroid chemical structures are similar to those of naturally-occurring hormones.

The theoretical correlation of chemical structure, possible binding sites and types of chemical bonding between carcinogens and tissue substances have been studied and several theories advanced by numerous investigators (Pullmann, 1955, 1964; Miller and Miller, 1961; Heidelberger, 1956).

Of particular importance is the question of perpetuation of the effects produced by chemicals altering mitotic activity with respect to the nucleus, and the cellular area of hydrocarbon attachment. Basically, it is a question of whether hydrocarbons attach in some fashion to the genetic apparatus of the cell directly, or to some regulating factor. Heidelberger (1964) disputes all evidence for DNA-hydrocarbon attachment on the basis of its chemical impossibility, and he has put forth the theory that carcinogenic hydrocarbons attach to cytoplasmic proteins concerned with mitotic regulation. His proposal and his hypothetical perpetuation circuits fit well with the evidence of Jacob and Monad on regulatory mechanisms (1961).

The data herein indicate the chemicals tested have: effects on rates of cell division, inhibition or acceleration; effect on multinucleation; slight morphological effects; and toxic effects. No amount of statistical analyses, including those in the present study, can fully support experimental data obtained from a series of in vitro studies (Schepartz, 1967).

Significance tests are necessary but not sufficient criteria for unquestionable acceptance of observed effects by one investigator; instead, the ability to reproduce data in different laboratories, under different conditions and by different investigators is of prime importance. Some comparative data are available for DMBA, but not for #742 or #837.

#742

Observed #742 effects included inhibition of mitosis, acceleration of mitosis, slight morphological changes and toxicity. The seemingly paradoxical mitotic effects, which would appear mutually exclusive, were observed at different concentrations of #742 (0.1 and 0.5 ppm) inhibited mitosis in the epithelial FHM line and similar inhibition was observed with fibroblastic RTG-2 line at 1.0 ppm (Table I). Inhibition of mitosis in normal cells but not malignant cells by carcinogens is a well documented occurrence (Vasiliev, 1963). The data herein indicate RTG-2 and FHM cells are "normal" in that malignant transformation has not taken place in control cultures. This conclusion coincides with direct observation of the cells and the impression that they are well organized, typical morphological types. However, in contrast to the effects of some chemical carcinogens on normal cells, the mitotic rate was not further reduced by higher concentrations of #742 but was increased.

At higher concentrations, the increment of mitotic increase was not the same in the two cell lines. The FHM line showed greater susceptibility

to the action of #742 at both concentration extremes. However, the lethal dosage of #742 for FHM was similar to that of RTG-2. Previous data on L-fibroblasts showed no reduction in rate of cell division at lower concentrations of #742. L-fibroblasts used in the experiments were highly anaplastic in comparison with RTG-2 fibroblasts. Tumors induced by #742 in vivo involved embryonic cells, or anaplastic cells to a greater degree than on normal cells.

Treated FHM cells had significant increases in multinucleation at low concentrations, but the percentage of multinucleation in RTG-2 fibroblasts deviated only slightly from that of the controls even at the highest nonlethal concentration.

Morphological changes were toward a more fibroblast-like cell. There was a reduction in the amount of cytoplasm and cells became slightly spindle shaped. This morphological alteration is possibly a result of reduced protein synthesis rather than induction of differentiation. Data obtained from studies on other mitotic inhibitory agents indicate alterations in protein synthesis (Alfred, 1965).

In comparison with DMBA and other carcinogens, Chemical #742 does not have typical carcinogenic action on normal in vitro cells. Its effects on normal RTG-2 fibroblasts was slight, whereas marked effects were noted on normal epithelial cells and anaplastic L-fibroblasts. The dual effects, inhibition and stimulation of mitosis, could possibly be interpreted as the initial action of this compound to inhibit cell division,

and secondly, the ability of this substance to cause in vitro malignant transformation (as opposed to DMBA). However, this theory was not confirmed by reinjection of #742 treated cells. There is little doubt as to its selective effects on anaplastic and epithelial cells. Jones (personal communication) observed chemical #742 effected noticeable change in epithelial cells of frog embryos before other cells were affected.

Chemical #742 treated L-fibroblast cell cultures were observed to have increased percentages of multinucleat cells up to concentrations effecting morphological changes (Bourne, 1964). At that time, it was concluded the increase resulted from abortive mitotic division and amitosis. Either of these procedures could account for increased multinucleation, especially since evidence of amitosis was observed. However, in the present study, it is felt that increased multinucleation occurred as a result of cell fusion to a greater degree than to abortive mitotic division or amitosis.

Positive correlation between the rates of cell division and the percentages of multinucleate cells can not be shown. All treated cultures showed significant increases in multinucleation with the exception of #742 treated RTG-2 fibroblasts which had only slight increases. Multinucleation is separate and independent from the effect on rates of cell division. Multinucleation could possibly be used as an indication of the degree of effect of hydrocarbons on cell membranes.

The foregoing does not rule out the possibility of amitosis and abortive mitotic division as a causal factor in multinucleation. Many

lobulated nuclei were observed in treated cultures which appeared as stages in amitosis. However, there is little doubt that fusion does occur in malignant in vivo and in vitro cells (Okada, 1965), and it is a logical consequence to expect a loss of normal membrane structure under the influence of surface active hydrocarbons.

#837

Chemical #837 has selective effects on epithelial FHM vs fibroblastic RTG-2 cells. Fibroblasts at low concentrations, below 0.1 ppm, were not inhibited whereas 0.3 ppm induced the cells to divide at a rate almost four times that of the controls. Cellular morphology of treated RTG-2 fibroblasts was markedly anaplastic with loss of fibroblastic configuration. Higher concentrations increased the mitotic index only slightly. Large numbers of "giant cells" characteristic of malignant cells in vitro were seen.

In contrast, FHM cells showed appreciable mitotic inhibition at 1.0 ppm #837 and marked inhibition at 5.0 ppm. Karyorrhexis and a breakdown of cell membranes were observed at this concentration and cells coalesced. The large percentage of multinucleate cells is thought due to this effect. No stimulation of mitosis by #837 was observed in FHM cultures.

On the basis of the staining reaction (uniform methods were used) chemical #837 treated cells did not pass the S stage (DNA synthesis) of interphase. Nuclei were pale and homogeneous. No stoppage of mitosis

in metaphase was noted. The cytoplasm was highly vacuolated, and indistinct cell membranes were observed in isolated cells. Increased multinucleation in RTG-2 fibroblasts is probably also due to both the effect of #837 on cell membranes causing coalescence and malignant transformation, as with chemical #742.

Data obtained here on epithelial cells is supported by Garner's observations (Garner, 1961) that cells on the surface of developing fish embryos exposed to #837 underwent coalescence and apparently ceased cell division.

DMBA

Effects of DMBA on fish cells closely paralleled that observed by Diamond (1967) on normal cells. RTG-2 fibroblasts responded to exposure to DMBA at much lower concentrations than epithelial FHM cells. Fibroblasts were inhibited at 0.5 ppm whereas epithelial cells were not noticeably inhibited until 1.0 ppm. The action of DMBA on both cell lines indicate again that these cells are nonmalignant. This contention is supported by Diamond's work (1967) in which she found malignant cells, including L-fibroblasts, not inhibited by DMBA but normal cells were.

In both cell lines, multinucleation increased as each line was inhibited. No evidence of coalescence in either line was noted. Toxicity was observed in the RTG-2 and FHM lines at approximately the same concentration.

Diamond (1967) has shown that DMBA does chemically bond with protein in both the cytoplasm and nuclei of nonmalignant cells; but not in malignant cells. She suggested insensitivity of malignant cells to DMBA's inhibitory effect as due to the failure of malignant cells to metabolize DMBA to a chemically reactive form. It was clearly shown by her work that entrance of the carcinogen into the cell was not impeded in malignant cells.

Alfred (1965) found as cell division was inhibited by DMBA during the first 24 hours growth, there was an increase in DNA per cell. This evidence indicates that cells lose control of the DNA regulatory mechanism at some point between S (DNA synthesis) and G_2. FHM cells particularly seemed to be fixated in the G_2 premitotic state in all treatment groups with viable cells.

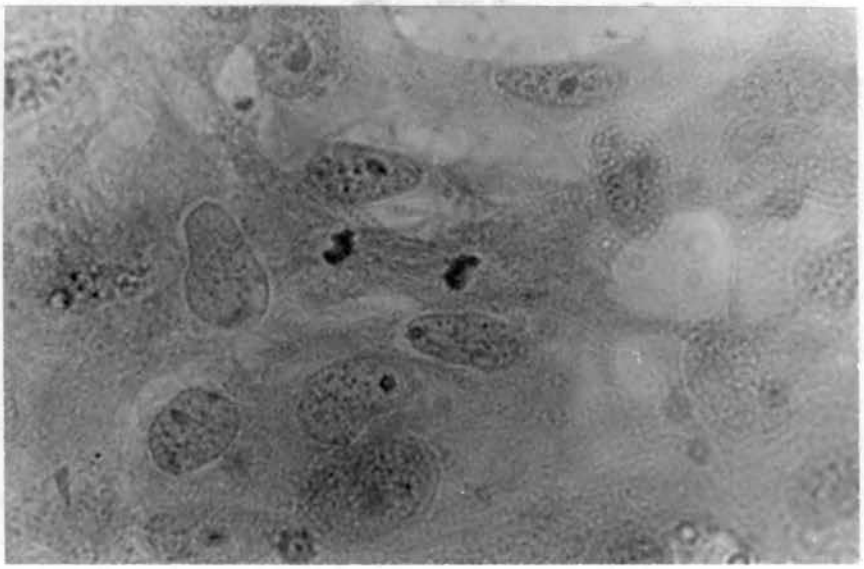

Figure 5. FHM Control, 1000 X

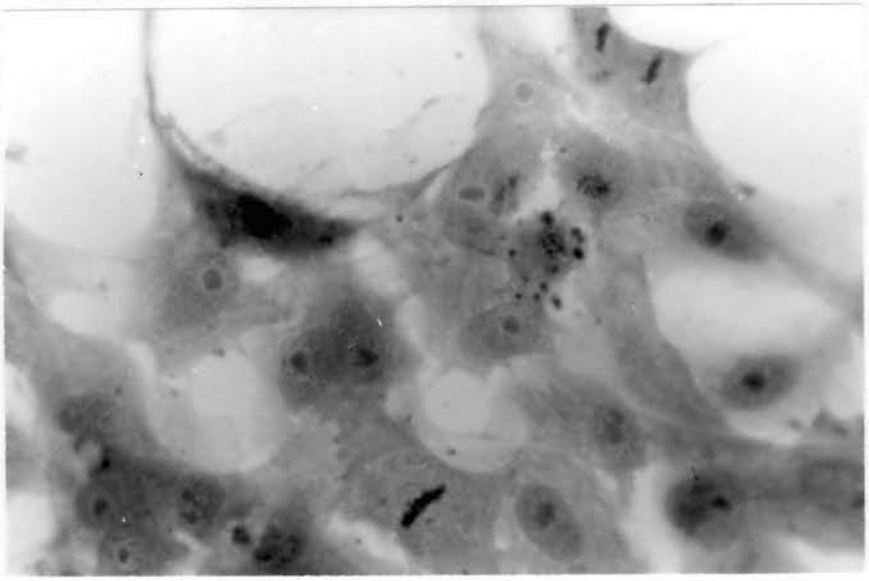

Figure 6. FHM, 1.0 ppm #742, 450 X

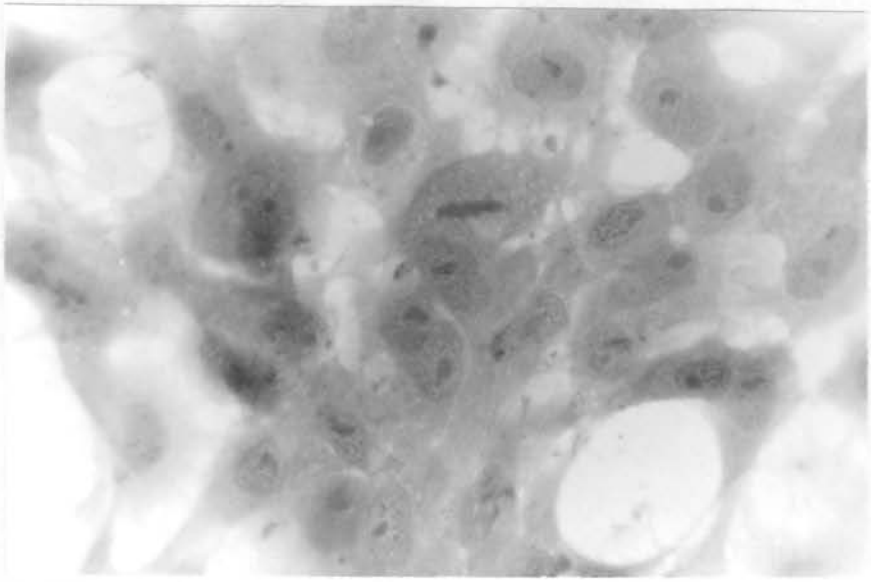

Figure 7. 5.0 ppm #742/FHM, 450 X

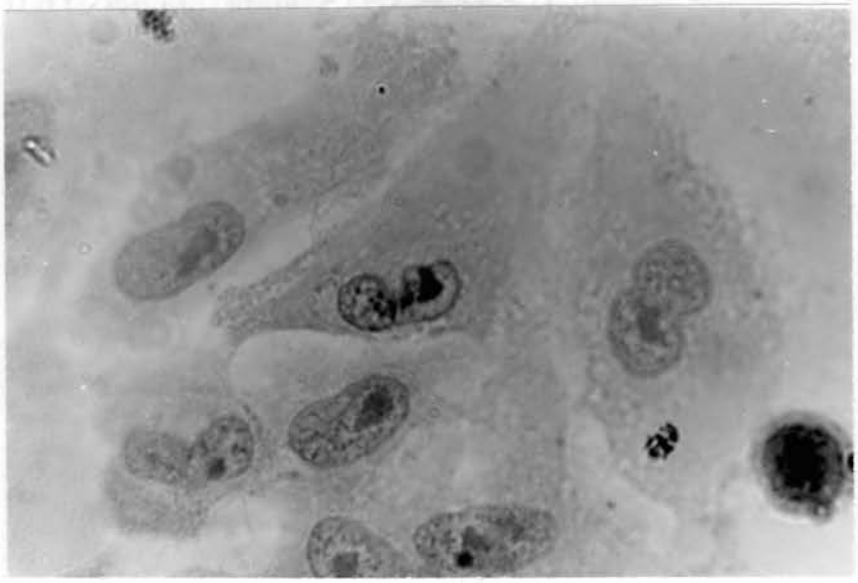

Figure 8. 5.0 ppm #837/FHM, 940 X

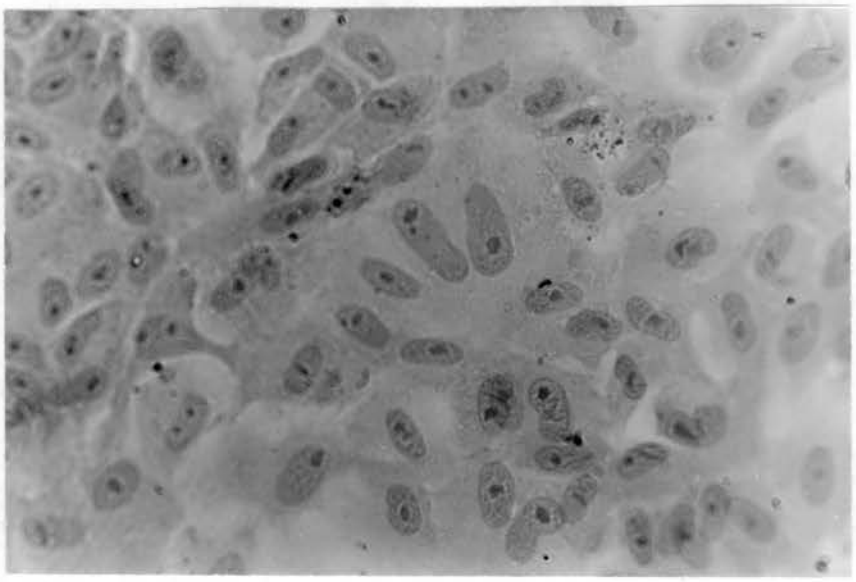

Figure 9. 5 ppm #837/FHM, 450 X

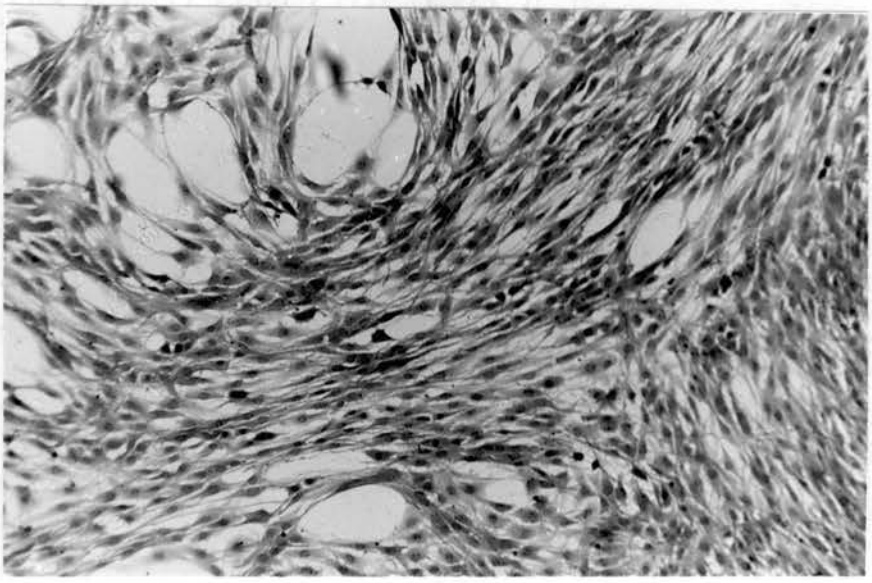

Figure 10. RTG-2 Control, 40 X

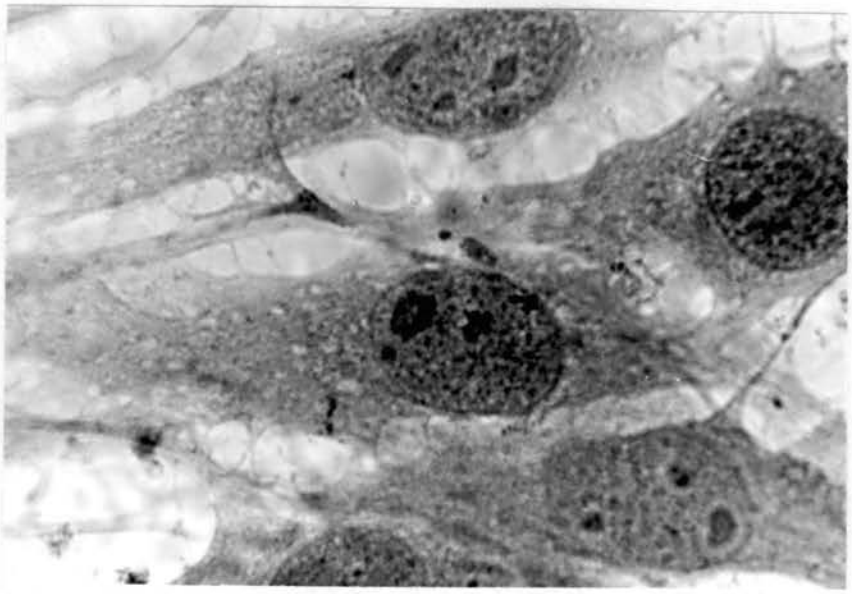

Figure 11. RTG-2 Control, 1000 X

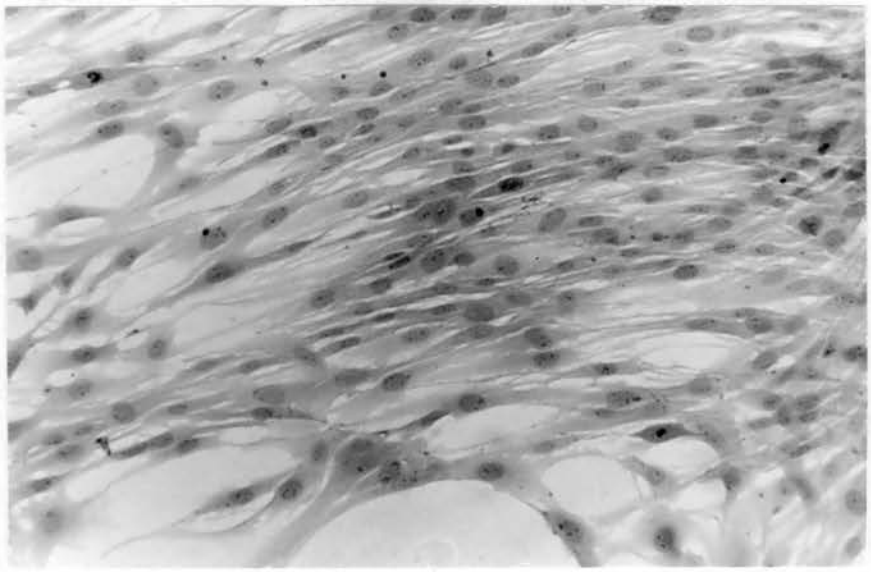

Figure 12. 1.0 ppm #742/RTG-2, 100 X

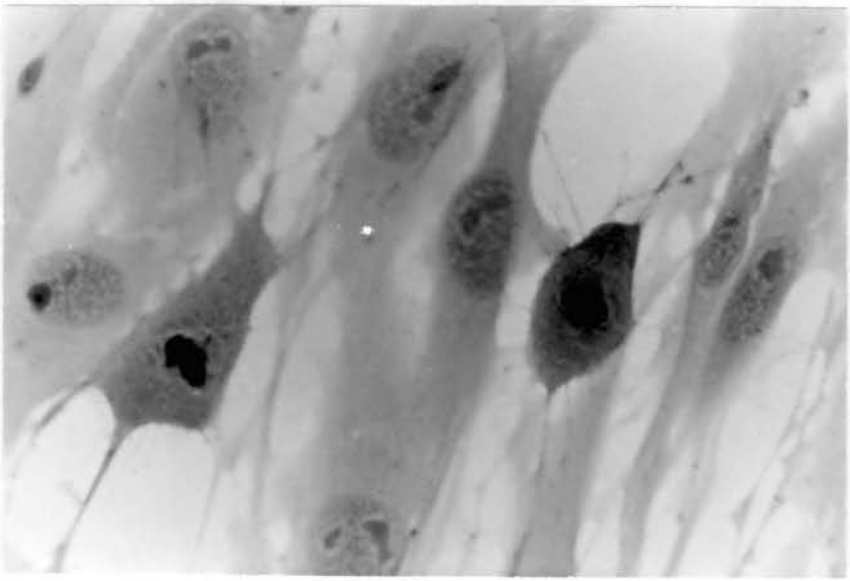

Figure 13. 5.0 ppm #742/RTG-2, 450 X

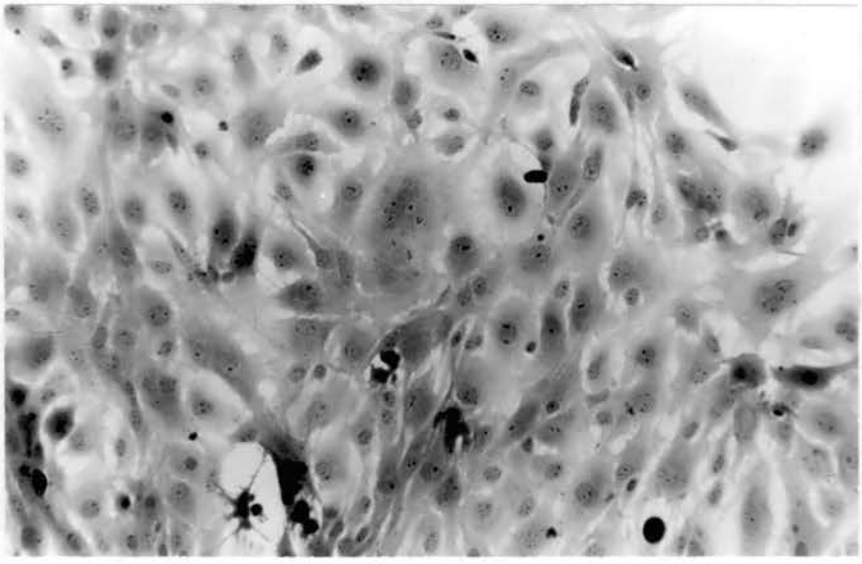

Figure 14. 0.3 ppm #837/RTG-2, 100 X

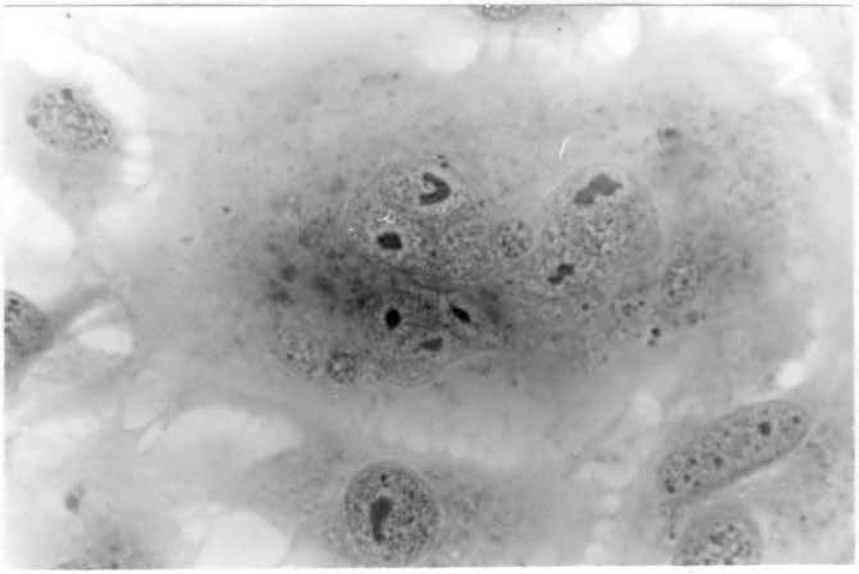

Figure 15. 0.3 ppm #837/RTG-2, 940 X

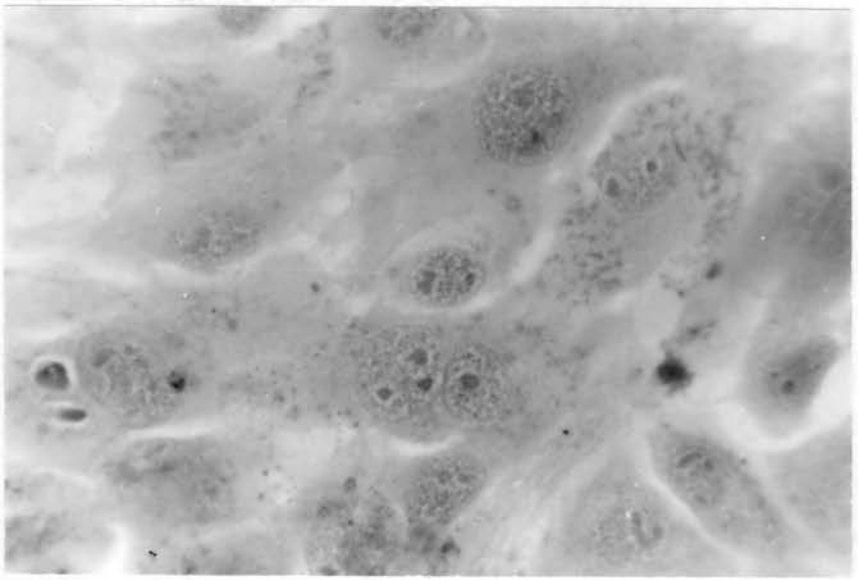

Figure 16. 0.5 ppm #837/RTG-2, 940 X

CHAPTER VI

SUMMARY AND CONCLUSIONS

The in vitro effects of three hydrocarbons, 1, 3, 5, (10), 16-estratetraen-3-ol (#742), 17α methylestra-1, 3, 5, (10)-triene-3, 17β -diol (#837), and 9, 10-dimethyl 1, 2, benzanthracene (DMBA or #788) were studied using two morphologically different fish cell lines, fathead minnow (FHM) and rainbow trout gonad (RTG-2).

Cells were grown directly on coverslips in Leighton tubes containing two milliliters medium and varying concentrations of experimental hydrocarbons from 0.1 ppm to lethal levels dissolved in propylene glycol. Experimental and control cultures were incubated at 21°C for seventy-two hours before fixation and staining in hematoxylin and eosin. Rates of cell division were studied and compared by determination of the mitotic indices. Morphological effects were studied and compared. Percentages of multinucleation were determined in all treatment groups.

Chemical #742 was found to slightly inhibit cell division (significant at the .10 level) in the epithelial FHM line at concentrations of 0.1 ppm; however this concentration did not inhibit line RTG-2. At a concentration of 1.0 #742, mitosis in cell line FHM was significantly increased (.01 level), but cell line RTG-2 was significantly inhibited (.01 level).

At 10.0 ppm, both cell lines showed toxic effects. Chemical #742 effected slight morphological changes in the epithelial FHM line, however none observed in the fibroblastic RTG-2 line. Percentage of multinucleate cells were only slightly changed in FHM line by this chemical, and no differences were observed in line RTG-2.

Previous studies on chemical #742 in this laboratory showed it to induce hyperplastic growths in the tail area of developing fish embryos when added to the water surrounding them, and the in vitro effects of this chemical on mammalian L-fibroblasts (considered by the author as a malignant cell line) studies in this laboratory showed the chemical to induce morphological changes, increase the mitotic index, and influence the percentage of multinucleate cells. Mitotic inhibition was not observed in L-fibroblasts treated with #742. On the basis of the in vitro effects of known carcinogenic hydrocarbons and similar data obtained in the present study, it is possible that chemical #742 is a carcinogenic hydrocarbon.

Chemical #837 inhibited cell division in the fibroblastic RTG-2 line at a concentration of 0.1 ppm (significant at .01 level in comparison with 1.0 ppm which inhibited the epithelial FHM line (significant at .001 level). Mitosis was stopped in line RTG-2 at 5.0 ppm and in line FHM at 10.0 ppm. Marked increases in the percentages of multinucleate cells were observed in RTG-2 and FHM lines at 0.1 and 1.0 ppm respectively. Previous observations in this laboratory on the in vitro effects of chemical #837 showed it to cause fading of cleavage lines in

in early fish embryos. It was observed in the present study that #837 caused cells to coalesce.

DMBA (#788), a known carcinogen, strongly inhibited mitosis in line RTG-2 at 0.5 ppm (significant at .001 level) and mitotic activity was stopped at 5.0 ppm. FHM cultures were slightly inhibited at 1.0 ppm (not statistically significant) and mitotic activity was stopped at 5.0 ppm. In both cell lines multinucleation was increased at the same initial concentration causing a reduction in the mitotic rate; however the percentages were decreased at concentrations causing stoppage of mitotic activity.

It was concluded that fish cell lines RTG-2 and FHM gave responses similar to those given by non malignant mammalian cells to carcinogenic hydrocarbons. The increased percentages of multinucleate cells was interpreted as the effect of the hydrocarbons on cell membranes causing them to lose structure and allow coalescence.

Selective and differential responses were detected in the two morphologically different fish cell lines to exposure to the hydrocarbons. Chemical #742 produced mitotic acceleration in line FHM at 1.0 ppm, but this same concentration inhibited the mitotic rate in line RTG-2. At 10.0 ppm #742, mitotic acceleration was observed in line RTG-2, but this same concentration was toxic to line FHM. Chemical #837 selectively acted upon line RTG-2 at low concentrations of 0.1 to 0.5 ppm to effect an increase in mitotic rate, and at 1.0 ppm it was toxic. However line FHM did not respond to low levels and was inhibited by

DMBA, however line RTG-2 responded at much lower concentration levels.

Statistically significant changes in the percentage of multinucleation were detected at concentration levels causing alterations in the mitotic rate.

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