THE EFFECTS OF URETHAN ON FISH EPITHELIAL AND FIBROBLAST CELLS IN VITRO

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CHAPTER I

INTRODUCTION

Research on urethan is not new. Ever since it was first found to be carcinogenic (Nettleship and Henshaw, 1943), much work has been done with this compound. However, to the author's knowledge no research has been done with urethan at the cellular level. Tissues have been examined histologically *in vivo* and different cell types have been studied using tissue explants *in vitro*. Therefore this problem was undertaken to see if the effects of urethan *in vivo* can be duplicated *in vitro*.

The paper is concerned mainly with the effects of urethan on mitotic rates and on cell morphology. Urethan is referred to as ethyl carbamate, urethane, and ethyl urethan in the literature. The author will only use the terms ethyl carbamate and urethan in this paper.

CHAPTER II

REVIEW OF LITERATURE

Urethan is an ethyl ester of carbamic acid with the formula $C_3H_7NO_2$ (Freese, 1965; Shulik and Hartwell, 1958). It is a colorless crystalline compound produced either by the action of ammonia on ethyl carbonate or by heating urea nitrate and ethyl alcohol. One gram of urethan is soluble in 0.5cc of water, 1.0cc of alcohol, or 3.0cc of glycerin. An aqueous solution of urethan is neutral to litmus (Neilson, Knott, and Carhart, 1960).

Urethan is carcinogenic to mice (Nettleship and Henshaw, 1943) in which it induces hepatomas and lung adenomas (Law and Precerutti, 1963) and lymphomas (Doell, 1962; Ito, Hoshino, and Sawauchi, 1965). It does not have an appreciable leukaemogenic action on adult mice (Berenblum and Trainin, 1960; Kawamoto, Kirschbaum, and Taylor, 1958), but induces leukaemia in newborn mice (Fiore-Donati et al., 1961; Berenblum, Boieto, and Trainin, 1966; Cividalli, Mirvish, and Berenblum 1965; Vesselinovitch and Mihailovich, 1966), and augments leukaemogenesis by X-rays (Berenblum and Trainin, 1960, 1961), estrogen or methylcholanthrene (Kawamoto, Ida, Kirschbaum, and Taylor, 1958). However, if one dose of fractionated whole-body X-radiation and bone marrow

cells are given one to eight weeks prior to a single dose of urethan, then the incidence of lung tumors decreases (Foley and Cole, 1964, 1966). Urethan is also carcinogenic to rats (Tannenbaum, Vesselinovitch, Matoni, and Stryzok-Mitchell, 1962), hamsters (Shubik et al., 1961), and fish embryos (Battle and Hisaoka, 1952).

Urethan produces chromosome damage in rat tissues. These effects on chromosomes are apparently specific for ethyl carbamate, since other homologues are inactive or less active (Berenblum et al., 1959). This is not to infer that other derivatives of urethan are not carcinogenic (Hueper, 1964).

When administered to rats, urethan was evenly distributed in tissues within a few hours, metabolized and largely excreted in the expired air as carbon dioxide, and only about 4% of the dose appeared in the urine in 24 hr (Boyland and Rhoden, 1949; Skipper et al., 1948).

It has been found that urethan decreases dehydrogenase activity in malignant tumor cells grown as explants in vitro (Black and Speer, 1953, 1954). It also decreases adenosine-triphosphatase activity in tissue homogenates (Gross, 1954) and increases the activity of certain oxidation-reduction enzymes in mice (Uzunov, 1964).

Urethan decreases the mitotic rate and finally kills chick fibroblast cells when a certain concentration of the drug is reached (Bastrop-Madsen, 1949; Geiersbach, 1939). However, in other tissue cultures it has been shown that

after a brief latent period the mitotic coefficient will at first increase and then decrease at a later time (Bucher, 1949). Also, epithelial cells in lung explants survive urethan treatment longer than the rest of the tissue (Globerson and Auerbach, 1965). Urethan also decreases the nuclear volume when tissue cultures are used (Gattiker, 1952).

When skin is treated with urethan *in vitro* it will cause homografts to last longer than if urethan was not used. This fact is observed because urethan causes the membranes to lose their antigens; thus, the typical antigen-antibody response that takes place in homografts does not occur (Bonmasser *et al.*, 1966). Also, mice treated with urethan and a sublethal dose of X-radiation suppressed the homograft response to a greater degree than that observed when mice were treated only with a sublethal dose of X-radiation (Cole and Davis, 1962).

Urethan damages the bone marrow and causes developmental abnormalities in the skeletal system of the rat (Ghera and Kaplan, 1964; Takacri, Tanobe, and Shimamoto, 1966). The liver and lung show a greater tumorigenic response to urethan than any other organs in infant mice (Klein, 1966). However, these are not the only susceptible organs because urethan is a multipotential carcinogen (Tannenbaum and Silverstone, 1958; Tannenbaum, 1963).

The development of urethan-induced thymic lymphomas in mice is augmented by gonadectomy in males. Also, more urethan-induced hepatomas occur in males than in females in

newborn mice. Thus, the presence or absence of tumors depends to some extent on the hormonal influence of the host (Liebelt, Liebelt, and Lane, 1964; Ito, Hoshino, and Sawauchi, 1966).

Schmahl (1964) reported that urethan acted as a carcinogen but not as a syncarcinogen. Schmahl reached this conclusion when he found that urethan and 9,10-dimethyl-1,2,-benzantracene both caused cancer in mice. However, when they were simultaneously applied there was no additive effect.

Many permanent cell lines have originated from cells of homiothermic animals (Paul, 1960), whereas few such lines have originated from cells of poikilothermic animals. Recently poikilothermic cell lines have been established from gonads of the fingerling rainbow trout, Salmo gairdneri (Wolf and Quinby, 1962), from fin tissue of the yellow-striped grunt, Haemulon flavolineatum (Clem, Moewus, and Sigel, 1961), from tissue posterior to the anus of the northern fathead minnow, Pimephales promelas (Gravell and Malsberger, 1965), and from tongue tissue of the bullfrog, Rana catesbeiana (Wolf and Quimby, 1964).

CHAPTER III

MATERIALS AND METHODS

In this project two cell lines were maintained. One was a fibroblast line established from gonads of the fingerling rainbow trout, Salmo gairdneri; the other an epithelial line taken from skin tissue posterior to the anus of the northern fathead minnow, Pimephales promelas. The fibroblast and epithelial cell lines are referred to as the RTG-2 and FHM cell lines, respectively. Both cell lines were obtained from Dr. Kenneth E. Wolf at the Eastern Fish Disease Laboratory in Kearneysville, West Virginia.

The cell lines were maintained as monolayers in milk dilution bottles. They were grown in a medium consisting of Eagle's minimum essential medium(84%), fetal bovine serum (10%), L-glutamine(1%), and an antibiotic mixture of penicillin-streptomycin(5%--250 units/ml). Nystatin(50 units/ml) was added whenever a fungus or mold contamination occurred. However, as soon as the fungus or mold contamination disappeared the Nystatin was removed because it was toxic to the cells (Roy W. Jones, Dept. Zoology, Okla. State Univ; personal communication).

Because of the growth of the cell cultures, they had to be diluted and transferred every two or three weeks. This

procedure was accomplished as follows: the growth medium was removed from the cells and a versene mixture plus a weak solution of trypsin(0.075%) was added. After the cells became detached from the glass, the cell population was divided into approximately three equal parts and centrifuged for ten minutes in the refrigerator. Then the versene mixture was poured off and the growth medium was added. The cell clumps were broken apart by pipetting the cells up and down several times. The cells and medium were then transferred to milk dilution bottles, and a neutral pH was obtained by using 5% carbon dioxide. The cells were incubated at 20 C until the next transfer.

All of the transfers and most of the other work involving the cell culture were performed in a sterile hood which was disinfected with isopropyl alcohol immediately before use.

Elaborate care was taken in cleaning the glassware. Before washing, it was placed in a Calgon and sodium metasilicate solution for 24 hours, and then in a strong solution of Clorox(665ml Clorox/20,000ml of water) for 24 hours. It was then washed under running tap water twice, and boiled in a weak solution of Calgon and sodium metasilicate(100cc Calgon and sodium metasilicate to 10,000cc water) for 20 minutes at 98^{o} C. When the glassware had cooled enough to handle with rubber gloves it was rinsed under hot running water twice, then under deionized water three times, and dried for 24 hours in the sterile hood. After the glassware

had dried, it was sterilized by being placed in the autoclave for 30 minutes at 270 F at 20 lbs pressure.

A 5% stock solution of urethan was prepared and sterilized by filtration through a Millipore filter. It was readily soluble in the growth medium. The urethan was obtained from Arthur H. Thomas Co. in Philadelphia, Pa.

In order to estimate which concentrations of urethan were lethal, the cells were grown on coverslips in Leighton tubes. At 0 hours, growth medium containing different concentrations of urethan was added. Controls were also run in which only growth medium was added. After 72 hours the coverslips were removed and the cells were fixed in 10% formalin, hydrated, stained with Harris' hematoxylin (15 minutes), dehydrated with alcohol, cleared with xylene, mounted on slides, and observed with a microscope. If cells adhered to the coverslips the cells were considered to be alive. If there were no cells on the coverslips, the cells were considered dead and that concentration of urethan was considered lethal(2.0%). This process was done with both cell lines(RTG-2 and FHM).

After the toxic concentration of urethan was ascertained, five different sublethal concentrations of urethan were prepared--0.3%, 0.6%, 0.9%, 1.2%, and 1.5%. These concentrations of urethan were made up in the growth medium. A control was also prepared containing only growth medium(no urethan). The cells were then grown on coverslips, treated with the various concentrations of urethan(including the

control), and stained with hematoxylin as described in the previous paragraph. Ten slides per treatment for each cell line were prepared in this manner making a total of 120 slides. With these slides, the rate of cell division was estimated by using the mitotic index (Paul, 1960). In this procedure, 1000 cells are selected at random on a slide. Of these 1000 cells, the number of nondividing and dividing cells are recorded. By dividing the number of nondividing cells by the total number of cells, the percentage of dividing cells is obtained. The rate of cell division is a mitotic index. Since 10 slides were used for each treatment, a total of 10,000 cells were counted for each treatment.

Cell numbers were also measured with a Coulter counter, model B. This instrument records the number of cells/ml by counting the cells as they pass through an orifice. In using the Coulter counter, the cells were removed from the dilution bottles and separated from each other by using a weak solution of trypsin(0.075%) and Versene. Six bottles were used, one for each treatment(no urethan, 0.3% urethan, 0.6% urethan, 0.9% urethan, 1.2% urethan, and 1.5% urethan). The cells were then centrifuged, the trypsin was poured off, and the appropriate concentration of urethan and medium was added. The cells were then separated into two equal parts for each treatment. Half of the cells were placed in a small 8 oz prescription bottle, allowed to settle into a monolayer, and incubated at 20 C. The other half of the cell population was counted immediately with the Coulter

counter. After 72 hours, the cells that were growing in the prescription bottle were again removed from the glass with trypsin and counted in the Coulter counter. By comparing the number of cells at 0 hours with the number of cells at 72 hours one can estimate cellular survival and multiplication. The Coulter counter experiment was performed only with the RTG-2 cells. Ten samples were made with each treatment so that a total of 120 samples were examined with the Coulter counter.

In order to estimate which concentrations of urethan were significantly different from the control and from each other, Duncan's Multiple Range tests and Student's "t" tests were performed (Steel and Torrie, 1960). Standard deviations were also computed on the observations within each treatment.

The effects of urethan on cell morphology were observed by staining the cells with Harris' hematoxylin. The same hematoxylin stained cells used in the mitotic index experiment were used for studying morphology.

Certain concentrations of urethan caused the cells to stop dividing. However, the cells were still alive. The Feulgan technique was used to determine if DNA was present in these nondividing live cells. In the Feulgan technique, the cells are fixed in Zenker's solution, stained with Schiff's reagent, and counterstained with fast green. The cytoplasm is stained green and the chromosomes and chromatin are stained violet or purple if DNA is present.

CHAPTER IV

RESULTS AND OBSERVATIONS

The results are divided into six different sections: the effects of urethan on RTG-2 cells as measured by the mitotic index; the effects of urethan on FHM cells as measured by the mitotic index; a comparison of the effects of urethan on RTG-2 and FHM cells as measured by the student's "t" test using the mitotic index data; the effects of urethan on RTG-2 cells as measured by the Coulter counter; the effects of urethan on cellular morphology of the RTG-2 and FHM cells; and the effects of urethan on the presence of DNA in the RTG-2 cells.

The Effects of Urethan on RTG-2 Cells as Measured by the Mitotic Index(Table I, Table II, and Figure 1)

Table I illustrates the following points: 0.3% urethan caused an increase in the rate of cell division; 0.6% urethan caused the cell division rate to decrease; 0.9%, 1.2%, and 1.5% urethan caused cell division to cease; and 2.0% urethan was lethal to the cells. A graphic representation of table I is shown in figure 1.

Duncan's Multiple Range test was used in order to estimate which treatments were significantly different from each other at the .05 level(Table II). In this table any two means not underscored by the same line are significantly different from each other. In order for the lines to be drawn in this manner the means must be ranked. Using this test it was noted the control and 0.3% urethan differed significantly in cell division rate.

The Effects of Urethan on FHM Cells as Measured by the Mitotic Index(Table III, Table IV, and Figure 2)

Table III shows that on FHM cells urethan caused an increase in the rate of cell division at low concentrations (0.3%); at higher concentrations of urethan(0.9%) the rate of cell division decreased; at still higher concentrations of urethan(1.2% and 1.5%) cell division ceased; and finally, 2% urethan was toxic to the cells. A graphic representation of table III is shown in figure 2.

The Duncan's Multiple Range test was also performed on the FHM cell line(Table IV). As in table II, the test indicated which treatments were significantly different from each other at the .05 level. For example, the cell division rate in 0.3% urethan was significantly higher than either the control or 0.6% urethan.

Comparison of the Effects of Urethan on RTG-2 and FHM Cells as Measured by the Student's "t" Test Using the Mitotic Index Data(Table V)

Student's "t" tests were made using the data in table V. The two cell lines were compared at the treatment levels of the control, 0.3% urethan, 0.6% urethan, and 0.9% urethan. Student's "t" tests could not be performed at the treatment levels of 1.2% urethan and 1.5% urethan because none of the cells were dividing. The treatment levels that are boxed in are significantly different at the .05 level. It is noted that the FHM cells continued to divide when treated with 0.9% urethan, whereas the RTG-2 cells did not.

The Effects of Urethan on RTG-2 Cells as Measured by the Coulter Counter(Table VI, Table VII, and Figure 3)

In testing the controls, normally more cells would be counted at 72 hours than at 0 hours(refer to materials and methods). However, in this experiment trypsin proved to be very toxic to the cells, and only 24.3% of the control cells survived after 72 hours. Nevertheless, the data collected are still valid under the following assumption: if a greater percentage of cells than 24.3% were recovered after 72 hours, then that particular concentration of urethan caused a greater cell division rate than that exhibited by the control. Conversely, if a lesser percentage of cells than 24.3% were recivered after 72 hours, then that particular

concentration of urethan caused less cell division than that exhibited by the control. However, another theory must also be considered. The fact that a greater percentage of cells than 24.3% were recovered after 72 hours may mean that particular concentration of urethan caused the cells to survive better than in the control medium in which there was no urethan. In other words, the differences in cell numbers that one obtains with a Coulter counter may indicate differences in cell survival and not cell multiplication. Nevertheless, the author tends to feel that the Coulter counter experiment indicates differences in cell multiplication rather than cell survival. Reasons for this view will be given in the next chapter.

Table VI indicates that the cell division rate increased when the RTG-2 cells were treated with 0.3% and 0.6% urethan. The cell division rate dropped below the control at 0.9% urethan, and continued to drop at 1.2% and 1.5% urethan. In table VI the means are recorded as the number of cells/100 cells that survived 0.075% trypsin for 72 hours instead of as the percentage of cells that survived 0.075% trypsin for 72 hours. The means are recorded in this manner because the analysis of variance to be performed for the Duncan's Multiple Range test required that the data not be listed as percentages since they were taken from a normal population. Figure 3 is a graphic representation of table VI.

Duncan's Multiple Range test was made on the Coulter counter data in order to estimate which treatments were significantly different at the .05 level(Table VII). The cell division rate was significantly different between the control and both 0.3% and 0.6% urethan. Also, cells treated with 0.9% urethan divided significantly more slowly than the control cells.

Standard deviations were calculated on the observations within each treatment. The standard deviations were as follows: control--2.2; 0.3% urethan--4.3; 0.6% urethan--2.4; 0.9% urethan--4.1; 1.2% urethan--1.5; and 1.5% urethan--1.0. All of the standard deviations were low.

The Effects of Urethan on Cellular Morphology of the RTG-2 and FHM Cells

There appeared to be two typical shapes in the RTG-2 cells. One was a triangular-shaped cell with long protoplasmic extensions; the other was spindle-shaped(Figure 4). The FHM cells assumed two basic shapes: a rectangular or polygonal shape(Figure 5) and a triangular-shaped cell(Figure 6). Cells can be seen at various stages of the mitotic cycle in figures 7, 8, 9, 10, and 11.

The concentrations of urethan did not change the basic shapes of the cells with one notable exception. Concentrations of 1.2% and 1.5% urethan caused many of the cells to lose most of their cytoplasm; a thick membrane appeared around the nucleus, and the nucleus became darker. In many

respects this aberrant type of cell looked like a small lymphocyte(Figure 12).

Another major effect of urethan on the cells was the appearance of vacuoles in the cytoplasm. This vacuolated appearance of the cells began at 0.6% urethan and became more pronounced as the concentrations of urethan increased (Figure 13). Urethan also caused some of the nuclei to become lobed and enlarged(Figures 14 and 15).

There appeared to be no difference in the effects of urethan on cellular morphology in the two cell lines.

The Effects of Urethan on the Presence of DNA in RTG-2 Cells

As discussed earlier, certain concentrations of urethan (0.9%, 1.2%, and 1.5%) were nonlethal, but caused the RTG-2 cells to stop dividing. By using the Feulgan technique, it was found that both the control cells and the cells that were treated with 0.9% urethan contained DNA. The possible implications of this phenomenon will be discussed in the next chapter.

CHAPTER V

DISCUSSION

The evidence from this research indicates that various sublethal concentrations of urethan can cause the rate of cell division to: (a) rise(0.3%), (b) fall(0.6% and 0.9%), or (c) cease(1.2% and 1.5%). Concentrations of urethan higher than 1.5% kill the cells in both the RTG-2 and FHM cell lines. Battle and Hisaoka (1952) also observed these phenomena when they observed that urethan caused epithelial hyperplasia in the teleost embryo, Brachydanio rerio. Hyperplasia was most evident on the ventral surface of the pericardial sac, the ventro-lateral trunk regions, and occasionally on the tail. Bucher (1949) reported that urethan caused the mitotic coefficient at first to rise and then drop in tissue cultures. He concluded that the actions of urethan depend on the dose, length of action, and biological cellular resistance. Haddow and Sexton (1946) reported that a derivative of urethan, phenylurethan, caused an increased mitotic count in the crypts of Lieberkuhn in mouse gut. Phenylurethan also caused the retardation of growth of certain tumors. Bastrop-Madsen (1949) found that concentrations of urethan ranging from 0.66% to 1.2% caused fibroblast cells to decrease in mitotic activity. Geiersbach (1939)

stated that urethan, when added to the culture medium, had an immediate fatal effect on chick fibroblast cultures in concentrations above 3%, when exposed longer than two hours. Also, 0.5% to 0.75% urethan caused an inhibition in the rate of cell division when exposure was limited to 70 hours.

This study has shown that epithelial cells were more resistant to urethan than fibroblast cells. Epithelial cells continued to divide in 0.9% urethan, whereas the fibroblast cells ceased to divide. Globerson and Aurbach (1965) observed that the epithelial cells of thymus and lung explants survived 1% urethan for four days; whereas the lymphocytes, alveolar tissue, and connective tissue underwent extensive necrosis.

As mentioned in the introduction, urethan is a carcinogen. The question arises, how does urethan cause some cells to undergo rapid and uncontrollable growth? For an answer one must look at the chemistry of urethan and the changes it undergoes in the organism. Boyland and Nery (1965) reported that when urethan was administered to rats, rabbits, and man, it was oxidized by a process of N-hydroxylation to yield N-hydroxyurethan and its N and O-acetyl derivatives. They believed that N-hydroxyurethan played an important role in urethan carcinogenesis, although the action is still obscure. Boyland Nery go on to state that the enhanced carcinogenicity of urethan as compared with other analogues (e.g., methyl, n-propyl, and n-butyl carbamates), may be due to the following: (1) urethan is

N-hydroxylated to a greater extent in the animal body; (2) urethan is metabolized more rapidly and thus presents a higher concentration of carcinogen to the tissues during a shorter time; (3) the specificity of the ethyl group. Freese (1965) agreed with Boyland and Nery when he stated that urethan induces chromosomal aberrations only because it is N-hydroxylated to N-hydroxyurethan.

The concepts of Boyland and Nery have been completely refuted by Kaye and Trainin (1966). These two investigators have indicated that N-hydroxyurethan is not as potent a carcinogen as urethan, and that 70% of an injected dose of N-hydroxyurethan labeled with C^{14} was converted to urethan. Kaye and Trainin have found further proof for their work by using a new drug called SKF-525A. This drug interferes with certain oxidation-reduction reactions taking place in the microsomal fraction of the liver and, in this regard, has been found to inhibit the dehydroxylation of N-hydroxyurethan to urethan. By using this drug, Kaye and Trainin observed that SKF-525A inhibited N-hydroxyurethan carcinogenesis.

Actually, a very small amount of urethan stays in the organism. When administered to rats, urethan is evenly distributed in the tissues within a few hours, metabolized, and largely excreted in the expired air as carbon dioxide. Only 4% of the dose is found in the urine after 24 hours (Skipper, et al., 1948).

Another interesting fact concerning the metabolism of urethan is that newborn mice metabolize urethan only 0.1 as

rapidly as adults (Cividalli, Mirvish, and Berenblum, 1965). This phenomenon helps to explain why urethan is more carcinogenic in newborn mice than it is in the adults.

Tannenbaum (1963) believed that the chemical changes that urethan undergoes in the organism is not of paramount importance in urethan carcinogenesis. He suggested that urethan acts as a carcinogen by somehow hastening the occurence of tumors which might spontaneously arise in the same site later in life rather than induction de novo.

In chapter IV of this paper, the author described the morphological effects of urethan on the two cell lines. Briefly, these effects included vacuolization of the cytoplasm and lobed and enlarged nuclei. Also, the higher concentrations of urethan(1.2% and 1.5%) caused the cytoplasm of some of the cells to disappear almost completely and the nucleus to develop a thick membrane so that the cells resembled small lymphocytes. Geirebach (1939) observed most of these phenomena when he worked with the effects of urethan on chick fibroblast cells. He reported that urethan caused the cells to be vacuolated and pyknotic. Also, there was "rounding" of the cells. Haddow and Sexton (1964) found that urethan caused epithelial tumor cells(Walker rat carcinoma 256) to revert to a fibrous structure, with spindle cells and abundant stroma. This study did not demonstrate any changes in the basic structure of the epithelial or fibroblast cells, but it should be noted that it was

concerned with "normal" cells whereas Haddow and Sexton worked with tumor cells.

The rate of cell division was measured by the mitotic index and the Coulter counter. The mitotic index is the older of the two methods. Minot (1908) was one of the first people to use this research tool when he investigated problems of growth in rabbit embryos. Self (1937) and Jones (1939) also used the mitotic index to study the development of fish embryos. More recently, Bourne and Jones (1964) used this method to study the effects of a steroid on mammalian L-fibroblasts in vitro.

The Coulter counter was first used to determine the number of red blood cells per milliliter (Maltern, Brackett, and Olson, 1957). Brecher, Schneiderman, and Williams (1956) reported that the accuracy and repeatability of results with the Coulter counter were of the order of 2%, as judged from duplicate counts and dilution curves. Their measurements were also carried out with red blood cells.

In this study the mitotic index was judged the more reliable of the two because in using the Coulter counter the effect of trypsin on cell survival made it difficult to ascertain whether or not the results obtained represent a true picture of the effects of urethan on the cells. However, the Coulter counter data agree with the data obtained by the mitotic index. Also, the observations within each treatment had a low standard deviation. The fact that the standard deviations were low suggests that the counts made

by the Coulter counter were consistent. Also, other authors have observed the same phenomenon. Chen et al. (1964) found that trypsin injured human amnion cells in vitro. Gori (1964) reported that if cell suspensions of animal tissues were left in contact with trypsin for more than nine minutes, the cells would begin to die due to cellular digestion by trypsin. Trypsin also injures L-strain cells in vitro (Han, Miletic, and Betrovic, 1966). If L-cells are left in trypsin concentrations of 0.05% or more, there will be a temporary inhibition of growth for 40 hours, after which time growth will continue at an impaired rate.

The Coulter counter does have many advantages over the mitotic index if adverse effects on cells in vitro could be avoided. First, the Coulter counter counted many more cells in much less time than the mitotic index method. Using the mitotic index 10,000 cells could be counted in about two hours. When the Coulter counter was used, approximately 200,000 cells were counted in about 30 minutes. Secondly, there is more human error when the mitotic index is used. Investigators often disagree as to what constitutes the beginning of prophase. Also, fatigue eventually causes a serious source of error in visual counting.

Certain concentrations of urethan(0.9%, 1.2%, and 1.5%) caused RTG-2 cells to stop dividing. However, the cells were still alive. When control RTG-2 cells and RTG-2 cells that were treated with 0.9% urethan were examined for the presence of DNA by using the Feulgan technique, it was found

that DNA was present in both instances. This probably indicates that whatever the reason for the cells not dividing, it is not because of an absence of DNA. In order for a cell to divide in mitosis, the DNA content must be doubled so that the daughter cells will have as much DNA as the mother cell (DeRobertis, Nowunski, Franciso, 1965). Thus, DNA is essential for cell division. One theoretical mechanism of urethan action is that it prevents cells from dividing by destroying DNA. However, the evidence presented here indicates that urethan does not act in this manner as DNA is still present in the nondividing living cells. It is possible that urethan may even cause an increase in DNA, as Uzunov (1964) reported that during the process of urethan carcinogenesis DNA production rose slightly.

CHAPTER VI

SUMMARY

The effects of urethan on RTG-2 and FHM cells were studied *in vitro*. By using the mitotic index and Coulter counter, it was found that 0.3% urethan caused an increase in the rate of cell division while higher concentrations (0.6%, 0.9%, 1.2%, and 1.5%) caused either a decrease in the rate or a cessation of cell division. Concentrations of urethan higher than 1.5% killed the cells. The mitotic index data also indicated that epithelial cells continued to divide at a higher concentration of urethan than did the fibroblast cells.

By using the Feulgan technique it was found that there were concentrations of urethan at which the cells did not divide, but were still alive and still contained DNA.

The morphological effects of urethan on the two cell lines were also investigated. These effects included vacuolization of the cytoplasm, lobed and enlarged nuclei, and in some cells the cytoplasm almost completely disappeared and the nucleus developed a thick membrane around it so that the cells resembled small lymphocytes.

When the Coulter counter was used, it was discovered that even small amounts of trypsin(0.075%) appeared to be extremely toxic to the cells.

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APPENDIX

TABLE I

MITOTIC INDEX DATA FOR THE RTG-2 CELL LINE

Concentration of Urethan	Per Cent in Mitosis	Number of Dividing Cells/ 1000 Cells
0.0% (control)	1.83	18.3
0.3%	2.69	26.9
0.6%	1.51	15.1
0.9%	0.00	0.0
1.2%	0.00	0.0
1.5%	0.00	0.0
2.0%	lethal	lethal

TABLE II

DUNCAN'S MULTIPLE RANGE TEST OF MITOTIC INDEX DATA FOR THE RTG-2 CELL LINE

Concentration of Urethan	0.9%	1.2%	1.5%	0.6%	0.0%	0.3%
Number of Dividing Cells/ 1000 Cells	0.0	0.0	0.0	15.1	18.3	26.9

TABLE III

MITOTIC INDEX DATA FOR THE FHM CELL LINE

Concentration of Urethan	Per Cent in Mitosis	Number of Dividing Cells/ 1000 Cells
0.0% (control)	2.38	23.8
0.3%	3.78	37.8
0.6%	2.78	27.8
0.9%	1.39	13.9
1.2%	0.00	0.0
1.5%	0.00	0.0
2.0%	lethal	lethal

TABLE IV

DUNCAN'S MULTIPLE RANGE TEST OF MITOTIC INDEX DATA FOR THE FHM CELL LINE

Concentration of Urethan	1.2%	1.5%	0.9%	0.0%	0.6%	0.3%
Number of Dividing Cells/ 1000 Cells	0.0	0.0	13.9	23.8	27.8	37.8

TABLE V

MITOTIC INDEX DATA FOR THE RTG-2 AND FHM CELL LINE

Concentration of Urethan	Number of Dividing Cells/1000 Cells in the RTG-2 Cell Line	Number of Dividing Cells/1000 Cells in the FHM Cell Line
0.0% (control)	18.3	23.8
0.3%	26.9	37.8
0.6%	15.1	27.8
0.9%	0.0	13.9
1.2%	0.0	0.0
1.5%	0.0	0.0
2.0%	lethal	lethal

TABLE VI

COULTER COUNTER DATA FOR THE RTG-2 CELL LINE

Concentration of Urethan	Number of Cells/100 Cells That Survived 0.075% Trypsin
0.0% (control)	24.3
0.3%	32.7
0.6%	32.9
0.9%	17.4
1.2%	5.8
1.5%	4.8

TABLE VII

DUNCAN'S MULTIPLE RANGE TEST OF COULTER COUNTER DATA FOR THE RTG-2 CELL LINE

Concentration of Urethan	1.5%	1.2%	0.9%	0.0%	0.3%	0.6%
Number of Cells/ 100 Cells That Survived 0.075% Trypsin	4.8	5.8	17.4	24.3	32.7	32.9

Plate I

Figure 1. Effects of Urethan on RTG-2 Cells Using the Mitotic Index Data.

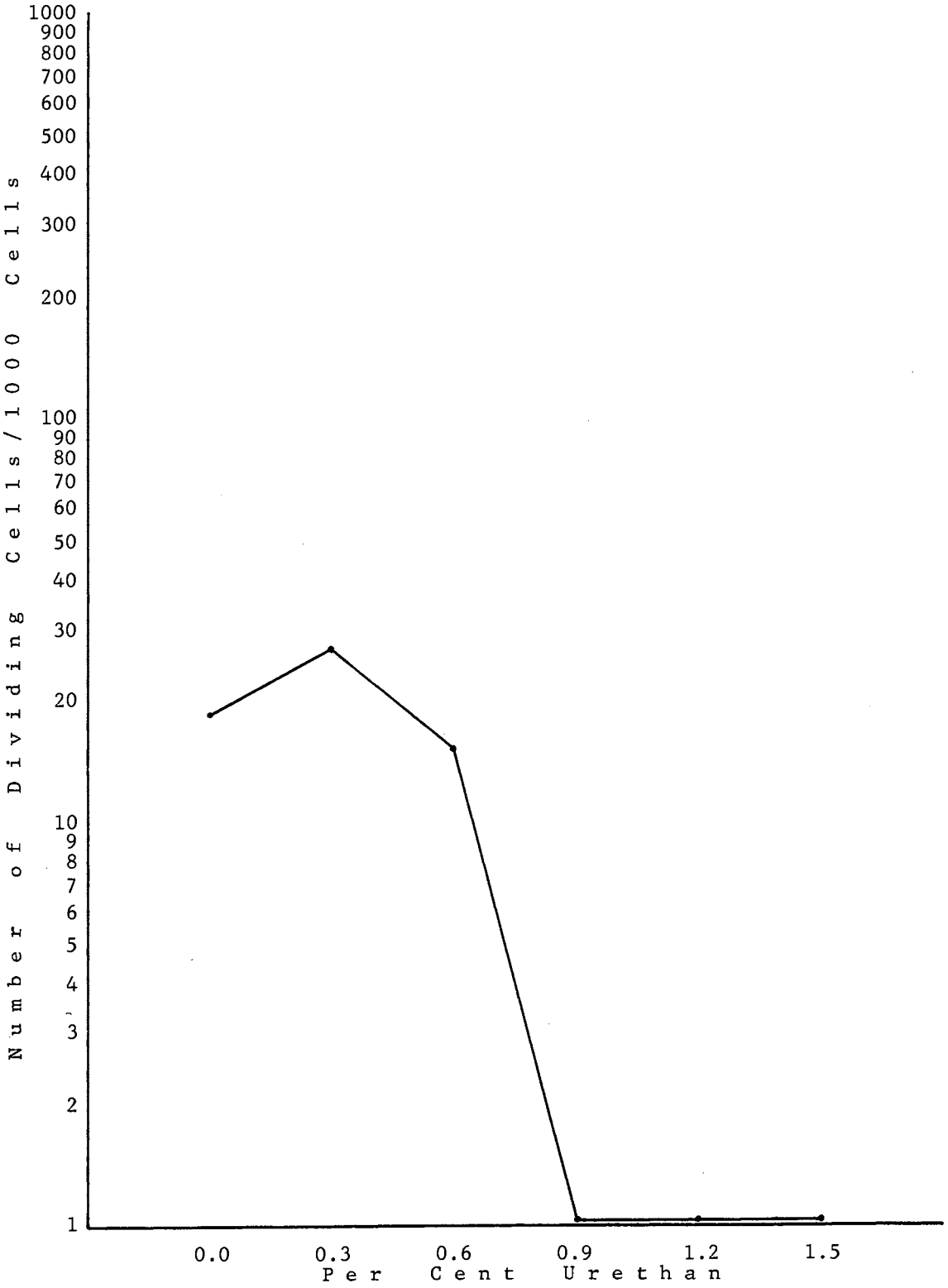
Number of Dividing Cells/1000 Cells
1000
900
800
700
600
500
400
300
200
100
90
80
70
60
50
40
30
20
10
9
8
7
6
5
4
3
2
1
0.0
0.3
0.6
0.9
1.2
1.5
Per Cent Urethan

Plate I

Plate II

Figure 2. Effects of Urethan on FHM Cells Using the Mitotic Index Data.

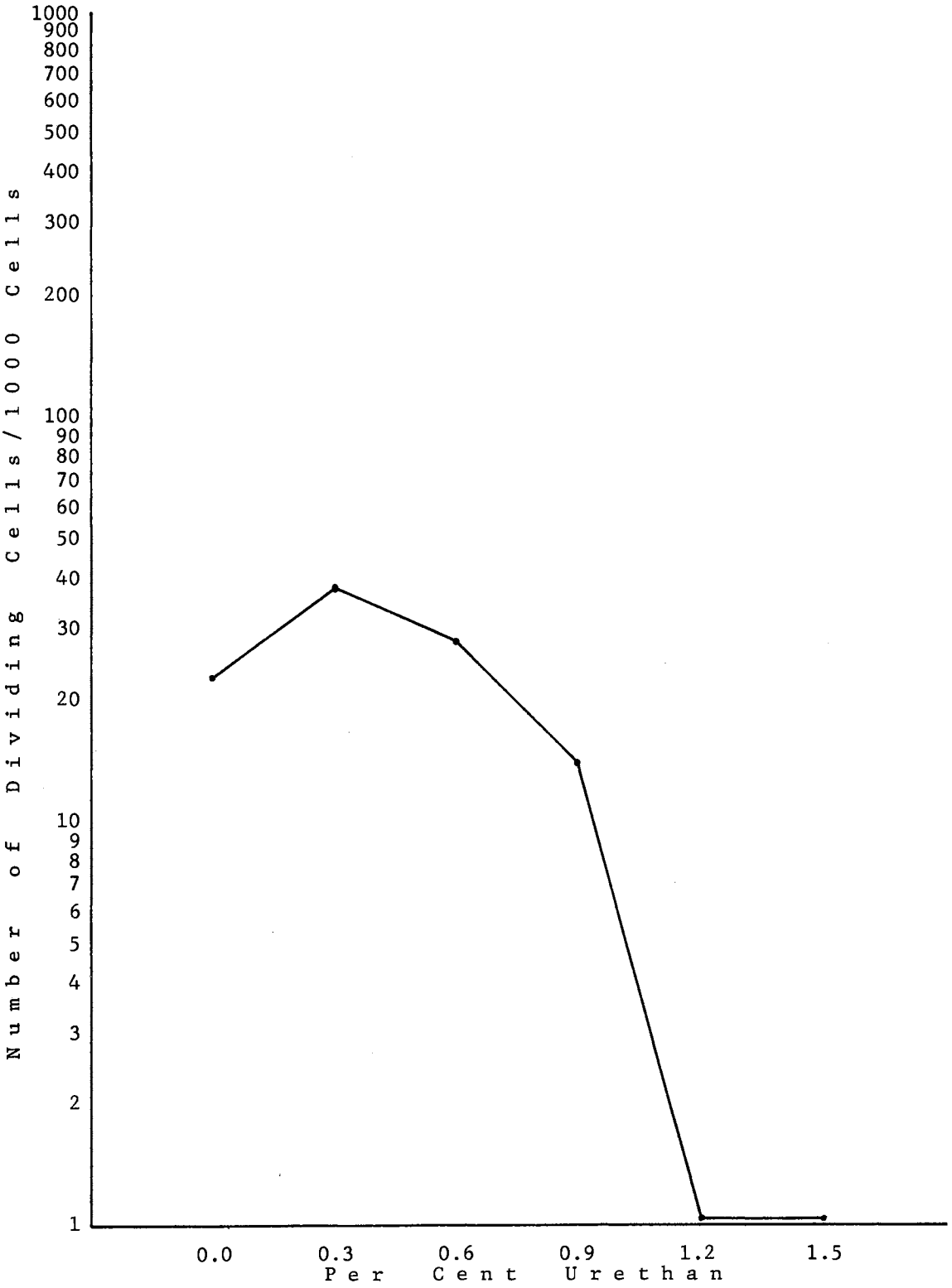
Number of Dividing Cells/1000 Cells
1000
900
800
700
600
500
400
300
200
100
90
80
70
60
50
40
30
20
10
9
8
7
6
5
4
3
2
1
0.0
0.3
0.6
0.9
1.2
1.5
Per Cent Urethan

Plate II

Plate III

Figure 3. Effects of Urethan on RTG-2 Cells Using the Coulter Counter Data.

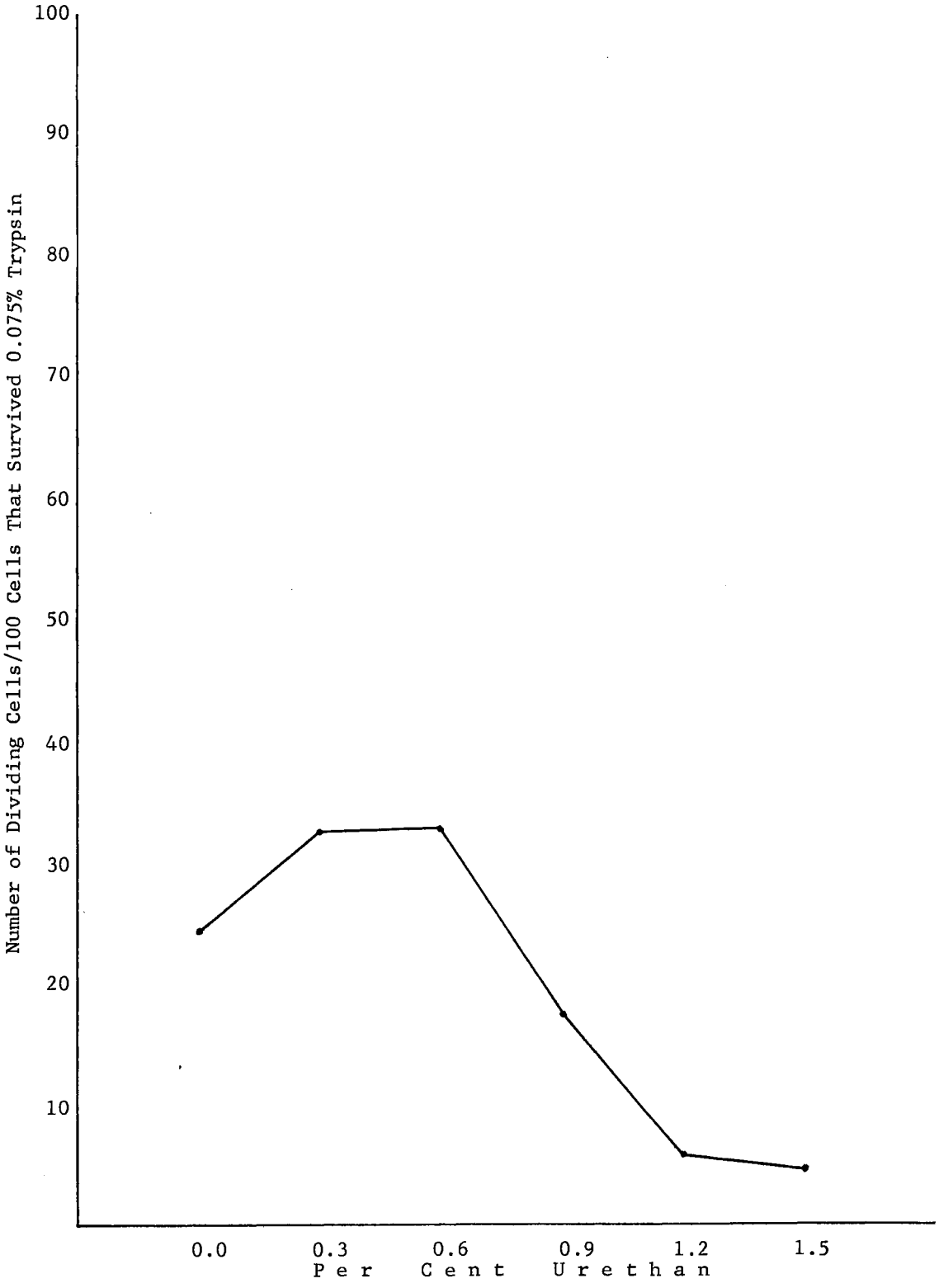
Number of Dividing Cells/100 Cells That Survived 0.075% Trypsin
100
90
80
70
60
50
40
30
20
10
0.0
0.3
0.6
0.9
1.2
1.5
Per Cent Urethan

Plate III

Plate IV

Figure 4. Typical Shapes of RTG-2 Cells.

5. A Typical Polygonal Shaped FHM Cell.

6. A Typical Triangular Shaped FHM Cell.

7. An RTG-2 Cell in Interphase.

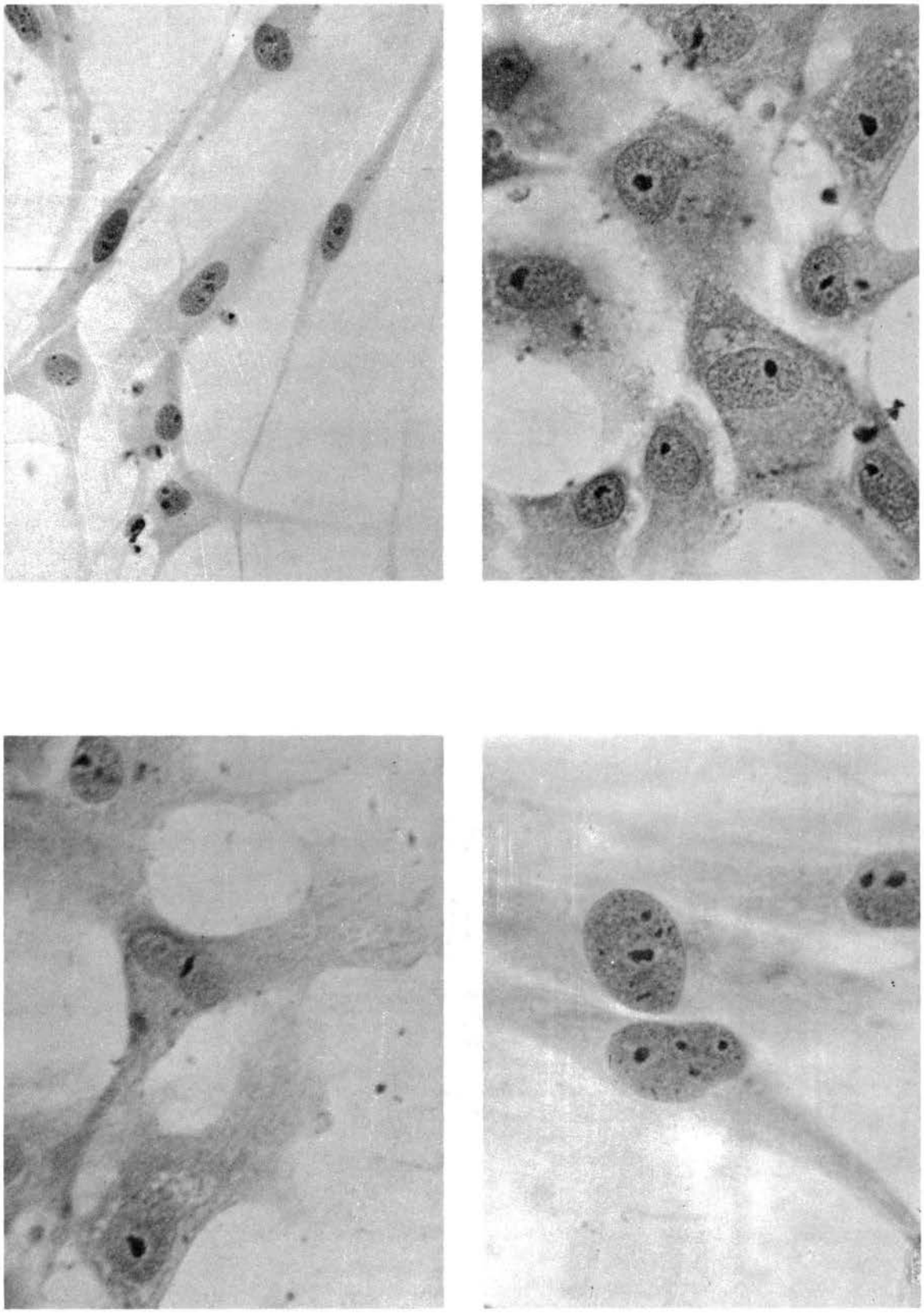

Plate IV

Plate V

Figure 8. An RTG-2 Cell in Prophase.
9. An RTG-2 Cell in Metaphase.
10. An RTG-2 Cell in Anaphase.
11. An RTG-2 Cell in Telophase.

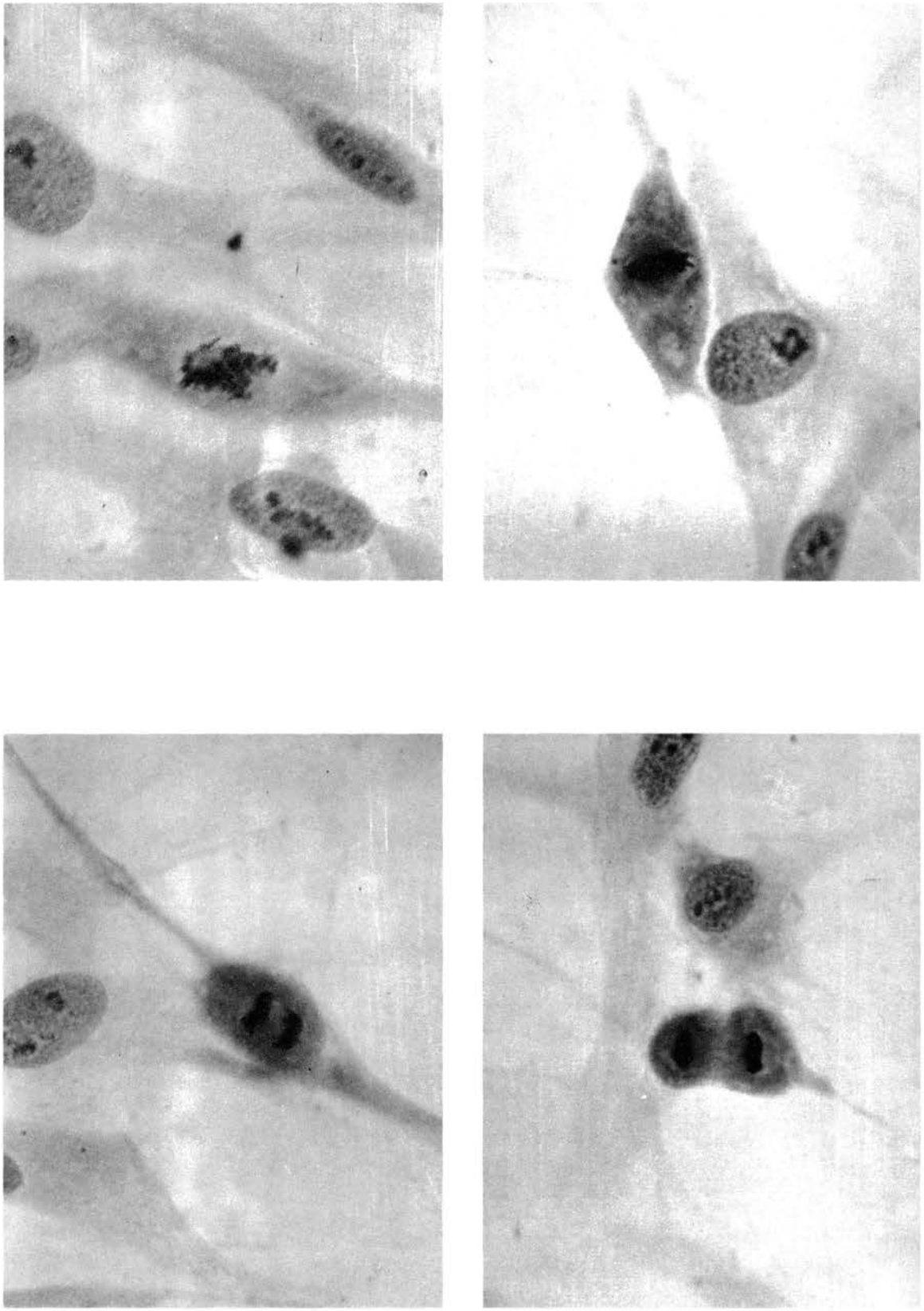

Plate V

Plate VI

Figure 12. RTG-2 Cells Exposed to 1.5% Urethan Showing the "Lymphocytic" Effect of Urethan.

13. RTG-2 Cells Exposed to 1.5% Urethan Showing Vacuoles in the Cytoplasm.

14. RTG-2 Cells Exposed to 0.6% Urethan Showing a Lobed Nucleus.

15. RTG-2 Cells Exposed to 0.6% Urethan Showing an Enlarged Nucleus.

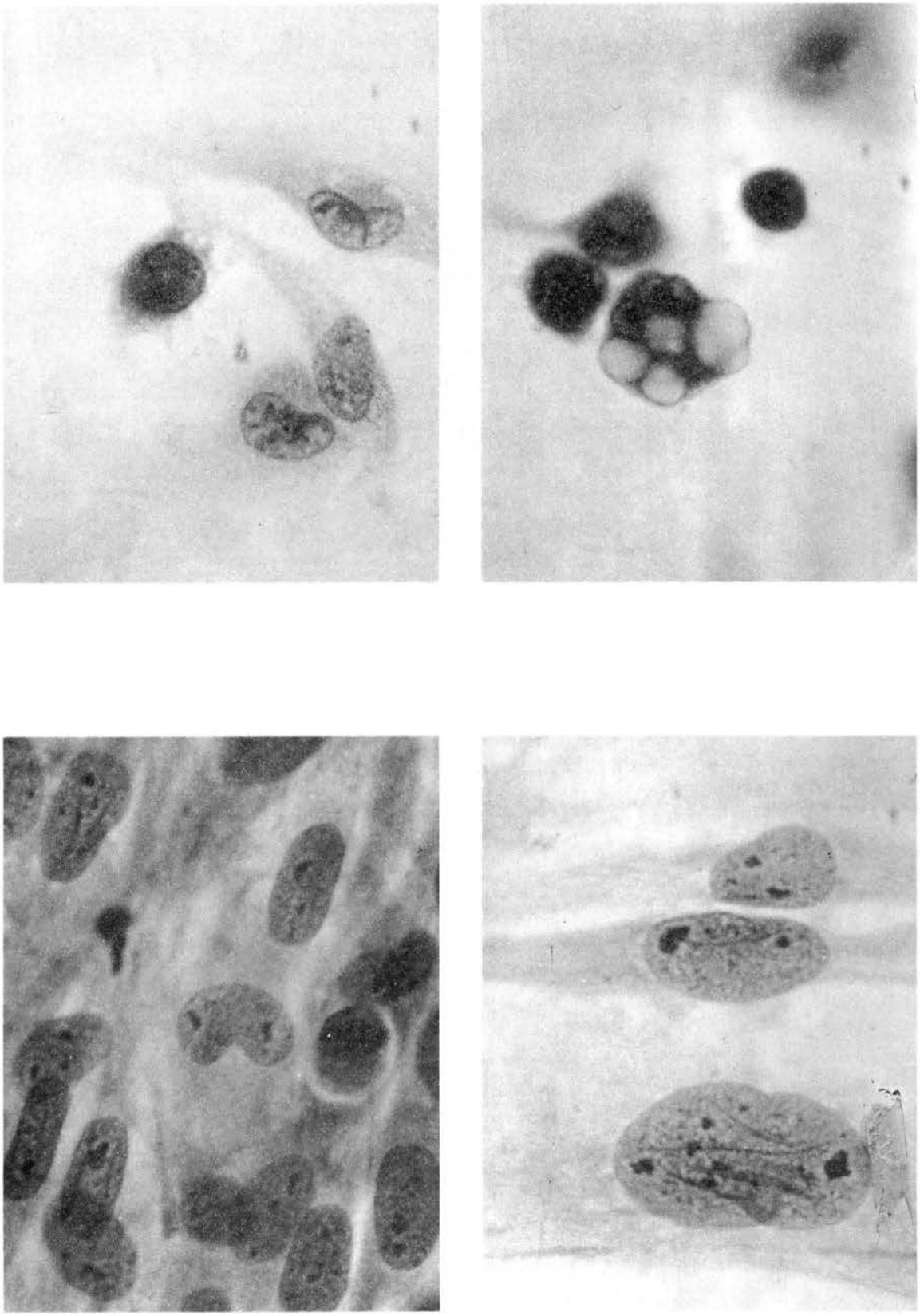

Plate VI

VITA

Leland Foster Morgans

Candidate for the Degree of

Doctor of Philosophy

Thesis: THE EFFECTS OF URETHAN ON FISH EPITHELIAL AND FIBROBLAST CELLS IN VITRO

Major Field: Zoology

Biographical:

Personal Data: Born in Stuttgart, Arkansas, June 30, 1939, the son of Mr. and Mrs. Mutt Morgans.

Education: Graduated from Stuttgart High School, Stuttgart, Arkansas, in May 1957; received the Bachelor of Science Degree from Texas Lutheran College in 1961, with a major in Biology and a minor in Chemistry; received the Master of Science degree from the University of Arkansas in 1965, with a major in Zoology; completed requirements for the Doctor of Philosophy degree at Oklahoma State University in July, 1968, with a major in Zoology.

Professional Experience: Graduate teaching assistant, University of Arkansas, 1964-65; graduate teaching assistant, Oklahoma State University, 1965-68.

Professional Organizations: Alpha Eta Chapter of Phi Sigma Society; Sigma Xi (associate member); American Association for the Advancement of Science.

Name: Leland Foster Morgans Date of Degree: July 31, 1968

Institution: Oklahoma State University

Location: Stillwater, Oklahoma

Title of Study: THE EFFECTS OF URETHAN ON FISH EPITHELIAL AND FIBROBLAST CELLS IN VITRO

Pages in Study: 47 Candidate for Degree of Doctor of Philosophy

Major Field: Zoology

Scope of Study: The purpose of this study was to determine the effects of urethan(ethyl carbamate) on two cell lines in vitro. Cells were grown as monolayers in milk dilution bottles. Most of the work in this paper is concerned with the effects of urethan on mitotic rates and cell morphology. However, the author also attempted to describe the effects of urethan on the presence of DNA in the cells.

Findings and Conclusions: Certain concentrations of urethan (0.3%) caused an increase in the rate of cell division while higher concentrations(0.6%, 0.9%, 1.2%, and 1.5%) caused either a decrease in the rate or a cessation of cell division. Concentrations of urethan higher than 1.5% killed the cells. Epithelial cells continued to divide at a higher concentration of urethan than did the fibroblast cells. The morphological effects of urethan included vacuolization of the cytoplasm, lobed and enlarged nuclei, and in some cells the cytoplasm almost completely disappeared and the nucleus developed a thick membrane around it so that the cells resembled small lymphocytes. There were certain concentrations of urethan at which the cells did not divide, but were still alive and still contained DNA.

ADVISER'S APPROVAL Roy W. Jones