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Leland F. Morgans
University of Arkansas at Little Rock

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The Effects of Urethan on Fish Epithelial And Fibroblast Cells in Vitro

Leland F. Morgans

Department of Biology

University of Arkansas at Little Rock

Little Rock, Arkansas 72204

ABSTRACT

The effects of urethan on RTG-2 and FHM cells were studied **in vitro.** by using the mitotic index, it was determined that 0.3 percent urethan caused an increase in the rate of cell division while higher concentrations (0.6, 0.9, 1.2, and 1.5 percent) caused either a decrease in the rate or a cessation of cell division. Concentrations of urethan higher than 1.5 percent 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.

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 oround it so that the cells resembled small lymphocytes.

INTRODUCTION

Research on urethan is not new. Ever since it was first found to be carcinorganic (Nettleship and Henshaw, 1943), much work has been done with this compound. However, to the author's knowledge no research with urethan has been done 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.** Also, the author wanted to ascertain if urethan had the same effect on fish cells as it did on mammalian cells.

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

Materials and Methods

In this project two cell lines were maintained. One was a fibroblast line established from gonads of 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 Kearneyville, 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 percent), fetal bovine serum (1 percent) L-glumatime (1 percent), and an antibiotic mixture of penicillin-streptomycin (5 percent — 250 units/ml).

Because of the growth of the cell cultures, they had to be diluted and transferred every two or three weeks. All of the transfers and most of the other work involving the cultures were performed in a sterile hood which was disinfected with isopropyl alcohol immediately before use.

A 5 percent stock solution of urethan was prepared and sterilized by filtration through a Millipore filter. It was readily soluble in the growth medium.

In order to estimate which concentrations of urethan were lethal, the cells were grown on coverslips in Leighton tubes. At O 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 percent 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 they 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 percent). 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 percent, 0.6 percent, 0.9 percent, 1.2 percent and 1.5 percent. 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 various concentrations of urethan (including the control) for 72 hours, 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 were selected at random on a slide. Of these 1000 cells, the number of nondividing and dividing cells are recorded. By dividing the number of dividing cells by the total number of cells, the percentage of dividing cells is obtained. The rate of cell division is a mitotic index. Snce 10 slides were used for each treatment, a total of 10,000 cells were counted for each treatment.

In order to estimate which concentrations of urethan were significantly different from each other and from the control, Duncan's multiple range test was performed on both cell lines (Steel and Torrie, 1960).

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.

Results and Discussion

Urethan does have an effect on the mitotic rate or RTG-2 cells (table I, table II). Table I illustrates the following points: 0.3 percent urethan caused an increase in the rate of cell division; 0.6 percent urethan caused the cell division rate to decrease; 0.9 percent, 1.2 percent, and 1.5 percent urethan caused cell division to cease; and 2.0 percent urethan was lethal to the cells.

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

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 lines to be drawn in this manner the means must be ranked. For example, by using this test it was noted that the control and 0.3 percent urethan differed significantly in cell division rate.

Urethan also had an effect on the mitotic rate of FHM cells (table III, table IV). Table III shows that on FHM cells urethan caused an increase in the rate of cell division at low concentrations (0.3 percent); at higher concentrations of urethan (0.9 percent) the rate of cell division decreased; at still hgiher concentrations of urethan (1.2 percent and 1.5 percent) cell division ceased; and finally, 2 percent urethan was toxic to the cells.

TABLE III

MITOTIC INDEX DATA FOR THE FRM 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

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 percent urethan was significantly higher than either the control or 0.6 percent urethan.

Thus, the evidence from this research indicates that various sublethal concentrations of urethan can cause the rate of cell division to rise, fall, or cease. Concentrations of urethan higher than 1.5 percent killed 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 ventrolateral 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 depended on the dose, length of action, and biological cellular resistance.

This study has also shown that epithelial cells were more resistant to urethan than fibroblast cells (table I and table III). Epithelial cells continued to divided in 0.9 percent urethan, whereas fibroblast cells ceased to divide. Globerson and Aurebach (1965) observed a similar phenomenon when they found that the epithelial cells of thymus and lung explants survived 1 percent urethan for four days; whereas the lymphocytes, alveolar tissue, and connective tissue underwent extensive necrosis.

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. The FHM cells also assumed two basic shapes, viz., a rectangular or polygonal shape cell and a triangular shaped cell.

The concentrations of urethan did not change the basic shapes of the cells with one notable exception. Concentrations of 1.2 percent and 1.5 percent 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.

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 percent urethan and became more pronounced as the concentrations of urethan increased. Urethan also caused some of the nuclei to become lobed and enlarged.

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

Geirebach (1939) observed many of these cellular aberrations when he worked with the effects of urethan on chick fibroblast cells. He reported that urethan caused the cells to become vacuolated and pyknotic. Also, there was a "rounding" of the cells. Haddow and Sexton (1964) found that urethan caused epithelial tumor cells to revert to a fibrous structure, with spindle cells and abundant stroma. The present 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.

Summary

The effects of urethan on RTG-2 and FHM cells were studied **in vitro.** By using the mitotic index, it was found that 0.3 percent urethan caused an increase in the rate of cell division while higher concentrations (0.6. 0.9, 1.2 and 1.5 percent) caused either a decrease in the rate of cell division or a cessation of cell division. Concentrations of urethan higher than 1.5 percent 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.

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