AN *IN VITRO* COMPARISON OF THE EFFECTS OF ETHYL CARBAMATE AND METHOXYETHYL CARBAMATE ON THE GROWTH AND CHROMOSOMES OF CHINESE HAMSTER FIBROBLASTS

By

NATHAN HOWARD COOK

Bachelor of Science
North Carolina Central University
Durham, North Carolina
1961

Master of Arts
North Carolina Central University
Durham, North Carolina
1963

Submitted to the Faculty of the Graduate College
of the Oklahoma State University
in partial fulfillment of the requirements
for the Degree of
DOCTOR OF PHILOSOPHY
May, 1972

AN IN VITRO COMPARISON OF THE EFFECTS OF ETHYL CARBAMATE AND METHOXYETHYL CARBAMATE ON THE GROWTH AND CHROMOSOMES OF CHINESE HAMSTER FIBROBLASTS

Thesis Approved:

Roy W. Jones
Thesis Adviser

L. Herbert Bureau

Milton R. Curd

Elizabeth T. Gandy

R R Walton

D D Durham
Dean of the Graduate College

ACKNOWLEDGEMENTS

I would like to express sincere appreciation to my major adviser, Dr. Roy W. Jones, for his immeasurable encouragement, understanding and guidance during the entire course of this study. I also extend acknowledgements to other members of my advisory committee: Drs. Elizabeth T. Gaudy, L. Herbert Bruneau, Milton R. Curd and Robert R. Walton. A very special appreciation is extended to Drs. Gaudy and Bruneau for serving as co-chairmen during the preparation of this manuscript in the absence of Dr. Jones.

Sincere gratitude is extended to: Dr. Lynn W. Gee, Microbiology Department, for extended use of the department's Coulter counter; Dr. Louise Higgins, Biochemistry Department, for suggestions and assistance with cell culture techniques; Dr. John H. Venable, Department of Anatomy, for assistance with techniques of light microscopy and autoradiography; Dr. John W. Thornton, for use of his laboratory facilities during his leave of absence; Mr. Donald Holbert, Department of Statistics for advice on the statistical treatment of some of the data; Union Carbide Corporation, New York, New York and Dan River Mills, Incorporated, Danville, Virginia for supplying samples and technical information on methoxyethyl carbamate; Mrs. Sharon Birch and Miss Sue White for typing the first draft of the manuscript; Mrs. Grace Provence for typing the final draft of the manuscript; and Mr. Eldon Hardy for preparation of all non-photographic figures.

A special acknowledgement is extended to my colleague and labora-

tory partner, Mr. Clinton H. Dixon for his technical assistance, encouragement and fruitful criticism during the entire study.

I wish to extend my very deepest and sincere appreciation to my wife Thelma, who with patience and understanding provided me with constant encouragement. A special thank you is extended to my two daughters, Carlene and Erika for seeming to sense the importance of the many hours I was absent from home. To you, Thelma, Carlene and Erika I am truly grateful.

Acknowledgements are also extended to the Ford Foundation, Oklahoma State University Research Foundation, and Barber-Scotia College for financial assistance during this study.

TABLE OF CONTENTS

LIST OF TABLES

Table | Page

LIST OF FIGURES

Figure Page

CHAPTER I

INTRODUCTION AND REVIEW OF LITERATURE

Research involving the carbamates, esters of carbamic acid, is not new. Many carbamates have been under scrutiny by researchers for a number of years, using *in vivo* and *in vitro* test systems. These statements are especially true of ethyl carbamate, also frequently referred to as urethan, urethane, ethyl urethan, and ethyl urethane. In the present study, the compound is referred to as ethyl carbamate (EC). The proliferation of studies, using carbamates as test chemicals, has probably stemmed from the early discovery that ethyl carbamate was a carcinogenic agent in mice, specific for the production of lung tumors (Nettleship and Henshaw, 1943; Henshaw and Meyer, 1944; Henshaw and Meyer, 1945; Larsen and Heston, 1945; and Larsen, 1947a). Prior to the discovery of its carcinogenicity, ethyl carbamate was used for many years as an anesthetic in man.

Ethyl carbamate was found to be carcinogenic in other test animals. Its carcinogenicity was observed in rats, where it produced lung tumors and hepatomas (Jaffe, 1947; Guyer and Clause, 1947a). However, the studies of later researchers did not confirm the presence of hepatomas in rats. Melanotic tumors were observed in hamsters (Oberman, 1965). Tannebaum and Silvertone (1958) and Tannebaum and Maltoni (1962) concluded that ethyl carbamate was a multipotential carcinogen and could produce many types of tumor.

Because of the carcinogenic activity of ethyl carbamate, other esters were tested and found to be carcinogenic in mice, but none were as effective as ethyl carbamate (Larsen, 1947b). A series of nitrogen-alkylated derivatives of ethyl carbamate was tested. Some of these derivatives demonstrated tumor producing activity, but again these compounds were not as effective as ethyl carbamate (Larsen, 1948).

In addition to exhibiting carcinogenic effects, ethyl carbamate has been reported to have a retarding effect on animal tumors (Haddow and Sexton, 1946; Huggins, et. al., 1947). The compound has been tested as a possible anti-neoplastic substance for various kinds of leukemia (Patterson and Haddow, 1946; Goodman and Lewis, 1946; Dustin, 1947; Kirshbaum and Lu, 1947; Creskoff, et. al., 1948; Watkins, et. al., 1948; Hirschboeck, et. al., 1948; Berman and Axelrod, 1948; and Hogreffe and Pedersen, 1950). For treatment of leukemia, many other carbamates have been tested (Skipper and Bryan, 1948; and Skipper, et. al., 1948).

Ethyl carbamate has been tested for its effects on mitotic activity and growth rates using *in vivo* and *in vitro* test systems. *In vivo* studies have indicated that the compound causes either inhibition or retardation of mitosis in various tissues (Dustin, 1947; Hohl, 1947; Guyer and Clause, 1947a, b; Kirshbaum and Lu, 1947; Green and Lushbaugh, 1949; and Wakonig-Vaartaja, 1964). *In vitro* studies have demonstrated that the compound causes inhibition of mitotic activity (Geiersbach, 1939; Battaglia, 1949; and Driessens, 1952), retardation (Bastrup-Madsen, 1949; and Patterson and Thompson, 1949), acceleration (Osgood, 1948), acceleration and retardation (Bucher, 1949a, b; Lasnitzki, 1949; Morgans, (1968).

The action of ethyl carbamate on the chromosomes has also been

tested on a number of biological systems, plants and animals. It was reported as the first chemical shown to produce structural chromosonal changes (Oehlkers, 1943). Oehlkers and Linnert (1949) tested the effects of ethyl carbamate along with potassium chloride, in plants, and found that it produced translocations in the meiotic chromosomes. Deufel (1951) demonstrated that treatment of Vicia faba root tips with ethyl carbamate-KCl mixtures caused breakage in the primary or secondary constriction region of the SAT chromosome. The small chromosomes broke more frequently in the median region. Deufel (1952) found that ethyl carbamate produced chromosomal aberrations such as translocations between chromatids or half chromatids, a few between quarter chromatids, and between entire chromosomes. Koller (1954) found that ethyl carbamate produced more breaks in the M (median centromere) than the S (subterminal centromere) chromosomes of Vicia and that it broke the M chromosomes preferentially at the secondary constriction.

Ethyl carbamate has produced a variety of effects on the chromosomes of animal cells. Its effects were tested on the bone marrow of mice and it was found to produce many abnormalities including bridging, clumping, and lagging (Rosin, 1951). Similar aberrations were reported in ethyl carbamate-treated Walker rat carcinoma 256 (Green and Lushbaugh, 1949). Fragments and anaphase bridges in the mitotic cells of the Walker rat carcinoma were reported (Boyland and Koller, 1954). Fragmentation, aggregation and other structural abnormalities were noted by the action of ethyl carbamate on the chromosomes of the grasshopper, Peocilocera picta (Rao, 1961). Chromatid and isochromatid breaks were demonstrated in mouse embryonic lung cells and Chinese hamster cells in vitro (Pogosyants, et. al., 1968). According to the

author's knowledge and a survey of the literature, no studies have been reported on the action of ethyl carbamate on the chromosome number of any species. Further, no reports have indicated whether the effects of the compound are cumulative or noncumulative, and reversible or irreversible.

Another carbamate, 2-methoxyethyl carbamate, has been used in the production of fabric finishing agents in industry. Research on this compound is relatively recent. Consequently, literature on the action of 2-methoxyethyl carbamate on biological systems (in vivo or in vitro) is virtually non-existent. In the present study the compound is called methoxyethyl carbamate or MEC. According to the author's knowledge, the only studies involving the compound have been conducted by its producer, Union Carbide Corporation (1967). In their studies, methoxyethyl carbamate and one of its derivatives, N, N-dimethylol-2-methoxyethyl carbamate, were tested for their ability to cause cancers in mice by injection. From their studies, it was concluded that methoxyethyl carbamate and its derivative did not increase the susceptibility to cancers in strains of animals which have a high incidence of the disease. It was further concluded that methoxyethyl carbamate should not cause cancer formation in humans. In the literature available to the author, no in vitro studies on the action of methoxyethyl carbamate on the growth of cell populations and chromosomes have been reported.

Since ethyl carbamate is a known and reliable carcinogenic chemical, and produces variable effects on growth and mitotic activity, and causes structural modifications in chromosomes, it would appear that its effects in vitro might possibly be used as a model for testing

other structurally related carbamates for parallel effects as probable carcinogens.

In this investigation, the effects of ethyl carbamate and methoxyethyl carbamate on the growth rates of cell populations and chromosomes of Chinese hamster fibroblasts, in vitro were studied and compared. Specific attempts were made to determine if the compounds exhibited a similarity or dissimilarity of effects on (a) growth rates of cell populations, (b) doubling time, (c) numberical and structural aberrations of the chromosomes, and (d) changes in cell populations as being reversible-temporary or irreversible-permanent.

CHAPTER II

MATERIALS AND METHODS

Characteristics of the Cell Strain Used

The Don strain of the Chinese hamster (Cricetulus griseus) lung was used in this study. Cultures of the strain were obtained from the Grand Island Biological Company, Grand Island, New York, hereafter referred to as GIBCO.

The strain has been characterized as being diploid, fibroblast-like, and of interest to investigators for use in virus, chromosome and autoradiograph studies. Also reported is the fact that these cells grow well in McCoy's 5a or Puck's N-16 medium, supplemented with 10% calf serum. The strain was isolated in September, 1962, from a normal eight-month old male adult Chinese hamster by Hsu (no publication). The cells have been reported to have a modal chromosome number of 22, with a stable diploid karyotype and a generation time of about 12 hours (American Type Culture Collection Repository, 1964).

The male Chinese hamster karyotype is reported to include 20 autosomes and two sex chromosomes. The autosomes include 14 metacentrics or submetacentrics and 6 subtelocentrics. The sex chromosomes include an X and a Y, both of which are reported as a medium-sized submetacentrics. The long arm of the X may show a weak secondary constriction at the distal third. The Y chromosome is cited as being morphologically unique (Hsu and Benirschke, 1967).

Establishment of Cultures

Several stock cultures were established and maintained as monolayers in 250 ml disposable screw-neck Falcon plastic flasks. For experimental work, monolayers were established in either 30 ml disposable screw-neck Falcon Plastic flasks or screw-cap culture tubes (GIBCO). All disposable plasticware (flasks and pipettes) used and subsequently mentioned was obtained from Falcon Plastics, Division of Bioquest, Los Angeles, California.

Stock and control cultures were grown in McCoy's 5a medium (Microbiological Associates, Bethesda, Maryland, hereafter called MA) supplemented with 10% fetal calf serum (GIBCO). The medium contained an antibiotic mixture; potassium penicillin G (50 mg/l), streptomycin sulfate (50 mg/l) and neomycin (100 mg/l). All cultures were incubated at 37°C.

Maintenance, Medium Changes and Transfers

The stock cultures were routinely replenished with fresh medium every two days. Transfers were made about every five days or when the stock cultures became confluent.

For the transfer procedure, the growth medium was decanted and the cultures rinsed with Puck's Saline A (PSA) obtained from GIBCO. The saline solution was decanted and the cultures were treated with trypsin: ethylene-diamine-tetra-acetic acid (EDTA) (GIBCO), 0.05% and 0.025% respectively, then reincubated for 5 minutes. After the cells became detached from surfaces of the flasks, they were aspirated vigorously with a sterile 10-ml serogical plastic pipette to break up cell

clumps. The cell suspensions were transferred to 12 ml conical, screw-cap centrifuge tubes and centrifuged five minutes at 500 to 850 rpm in an International Clinical Centrifuge, model CL (International Equipment Company, Needham Heights, Massachusetts). The supernatants were discarded and fresh growth medium was added to the cell buttons. The cells were resuspended with Pasteur pipettes. The suspensions were divided into halves or thirds, and pipetted into new flasks (250 ml). Excess cell suspensions were discarded. To each of the new flasks growth medium was added to adjust the volume to 10 ml. The cultures were gassed with air containing 5% CO_2, capped, labeled and incubated. Gassing was a routine procedure used for the establishment of all initial cultures. A log of medium changes and transfers was maintained.

To alleviate the possibility of contamination, all transfers and other procedures involving open cultures, for continued study, were done in a sterile hood. The hood was equipped with a U-V germicidal lamp which was left on continuously when cultures were not being changed, seeded or transferred. The hood was cleansed with 70% isopropyl alcohol prior to being used as an added precaution against contamination.

In order to facilitate pipetting, when work was done in the hood, pieces of rubber tubing were attached to the pipettes.

Cleaning and Preparation of All Non-disposable Culture Materials

Extreme care was taken in the cleaning and preparation of all materials, other than the disposable plasticware, for usage in the culture procedures. Immediately after usage, all glassware was soaked

and stored in plastic buckets containing a 0.5% aqueous solution of Microsolve tissue culture detergent (MA). Bottle caps, rubber policemen, syringes, Swinney filter holders and silicone stoppers were rinsed and stored in containers of deionized water.

The procedure for the washing of glassware, in the order of occurrence, included: scrubbing in a warm 0.5% Microsolve solution, rinsing three times in hot running tap water, rinsing in two changes of deionized tap water and rinsing in two changes of 2X deionized, glass-distilled water. The other materials were usually boiled in deionized water for one to two hours, then taken through the water rinses mentioned above.

The glassware was dried in an oven at 250°F, for one to two hours, cooled and wrapped either in double layers of aluminum foil or in sterilizing bags. The other materials were air-dried before being wrapped. All materials were autoclaved 20 minutes at 121°C and 18 psi.

Harvesting Cultures for Seedings and Cell Counts

The harvesting procedure for stock cultures used in seeding experimental flasks or tubes and for making counts of cell populations from terminal cultures were the same. Harvesting, in these cases, was similar to the transfer procedure previously mentioned. In this procedure, cells were resuspended in Hanks' Balanced Salt Solution (HBSS) purchased from GIBCO. Once the suspension appeared to be evenly dispersed and free of clumps, a 0.2 ml sample was withdrawn using a sterile 1-ml plastic pipette. The sample was added to a vial containing 19.8 ml of a filtered 0.9% NaCl solution (counting fluid).

All counting solutions (0.9% NaCl) were filtered through a Milli-

pore filter (0.45μ) and stored in 500-ml bottles. The filters were obtained from Millipore Corporation, Bedford, Massachusetts, hereafter called (MC). For dispensing the solutions into the vials a 10-ml Cornwall auto pipette (A. H. Thomas Company, Philadelphia, Pennsylvania) was used. The vials were filled with 20 ml of the counting fluid and 0.2 ml was removed to achieve a final volume of 19.8 ml.

The above mixture (cells suspended in the counting fluid) was aspirated 5 - 10 times and counted with a model B Coulter Counter (Coulter Electronics, Hileah, Florida). Five counts of the suspended sample were made and averaged. Five similar counts, using 19.8 ml of the counting fluid (without suspended cells), were made and averaged to give a background count. The average background count was deducted from the average cell count. The remainder was multiplied by the dilution factor. The product gave an estimate of the number of cells per ml. The number per ml was multiplied by the total volume of the suspension from which the sample was taken. The product provided an estimate of the number of cells per total volume of the suspension. The above techniques for harvesting and making cell counts were routinely used throughout the study when cell populations were enumerated with the Coulter Counter.

Based on the aforementioned estimates, cell suspensions of equal volumes were seeded into their respective culture vessels. These estimates were also used for terminal cultures in enumerating the number of cells in a population.

Preparations of Stock Solutions of the Compounds

A 10% (w/v) stock solution of each of the two test compounds,

ethyl carbamate (Nutritional Biochemicals Corporation, Cleveland, Ohio) and methoxyethyl carbamate (Union Carbide Corporation, New York, New York) was prepared by dissolving the compounds directly in McCoy's 5a medium. Both compounds dissolved readily in the medium. The pH of each solution was adjusted to 7.0 with 1N HCl or 1N NaOH. Each of the solutions was sterilized by passage through a sterile 0.45μ Swinney filter (MC). The two stock solutions were stored in a refrigerator at about 4-6^{o}C until they were used. Fresh stock solutions were prepared about every two weeks in an effort to avoid the possibility of chemical degradation due to long-term storage in solution.

In preparing the various concentration levels to be tested, measured volumes of each of the stock solutions were added to growth media containing 10% fetal calf serum to produce the desired final volumes and concentrations.

Growth Rate Studies

To establish the effects of the two compounds on the growth of Chinese hamster fibroblasts and to select concentrations for further study, several experiments were conducted. The design of these experiments was a modification of a procedure used by the Cancer Chemotherapy National Service Center (1962).

In each experiment, 18 flasks (30 ml) were seeded with equal volumes of a cell suspension to establish cultures. The cultures were incubated 18-24 hours to allow the suspended cells to settle and attach to the surfaces of the flasks. Six cultures were selected at random, harvested, and counted. An average of the number of cells in the six flasks was used as a baseline (number of cells at 0 time).

From the average an assumption was made that each of remaining cultures (uncounted) contained, on the average, the same number of cells.

The remaining cultures (12) were divided into four groups, with each group containing three cultures, i.e., one control culture and two experimentals. The growth medium from these cultures was decanted and replaced. The controls, 4 cultures, were treated with growth medium without either of the compounds. The four groups of experimental cultures were treated with growth media containing 30, 300, 3000 and 30,000 μg/ml of one of the compounds respectively. The cultures were incubated for 72 hours.

The cultures were then harvested and the cell populations were counted. For each concentration level the numbers of cells in the two cultures were averaged. The numbers of cells in the four control cultures were also averaged.

To determine the effect of the different concentrations of the test compounds on the growth rate of the cultures the following formula was used:

$$\frac{T - (Co)}{C - (Co)} = y \text{ (Growth Ratio)}, \text{ or } \frac{\text{Treated}}{\text{Untreated Control}}$$

The mean of the experimental cultures (T) for each dilution (dose) minus the mean of the baseline (Co) divided by the mean of the control (C) cultures minus (Co) gave the growth ratio (Y) at each dose level.

The growth ratio for the control (1.0) was taken as a constant or 100%. A growth ratio that exceeded 1.0 was taken as an accelerated growth rate. A growth ratio that was less than 1.0 indicated retardation or a decreased growth rate. A negative growth ratio coupled with microscopic observations indicated that the effects of a treatment were lethal or partially lethal. When a negative growth ratio was

obtained, the percentage of lethality was calculated. To calculate the percentage of lethality for a dosage level, the baseline number of cells (time 0) was divided into the mean number of cells at 72 hours. The remainder was then multiplied by 100 to give the percentage of lethality.

This experiment was done four times using ethyl carbamate and three times for methoxyethyl carbamate at the dosage levels indicated above. Similar studies (one for each compound) were done with the two compounds at concentrations of 6000,9000,12,000 and 15,000 μg/ml.

Secondary to the enumeration of cells in each of the cultures at 72 hours for effects on growth, all treated cultures were observed microscopically and compared with the controls. Observations were made on attachment of cells to the surfaces of the flasks, cell morphology and state of confluency. The pH of the media for all cultures was also noted and determined by changes in the color of the phenol red indicator (pH range 6.4 - 8.4). If the indicator retained its original color (red) or became slightly purple (alkaline), the concentration level was considered inhibitory or lethal. Changes of color to orange or yellowish indicated retardation and no prohibition of normal metabolism respectively when compared with controls. A change of the indicator from red to yellow was taken as a normal event, or as meaning that in media with or without a test compound nothing prevented the normal change of color due to acid production. These secondary observations were modifications of a procedure described by Toplin (1959).

Based on the results of the studies mentioned above, two concentrations (300 and 3000 μg/ml) for each of the compounds were selected for further study.

Doubling Time Experiments

Two experiments were done to establish and compare the effects of the two selected concentrations (300 and 3000 µg/ml) of each test compound on the doubling time of cell populations. In each experiment, the same concentration levels of the compounds were used. Culture tubes instead of the 30-ml plastic flasks were used in these experiments. The tubes were found easier for handling purposes and eliminated the need to transfer cell suspensions from flasks to centrifuge tubes for centrifugations. All centrifugations were done in the culture tubes.

In each experiment, 30 tube cultures were established in the usual manner and incubated 18-24 hours at 10° angle on a tissue culture rack (MA). Six cultures, selected at random, were harvested and counts of the cell populations were made. The average number of cells in the six cultures was used as a baseline (number of cells at time 0). The 24 remaining cultures were treated with growth medium or media containing the compounds, i.e., ethyl carbamate or methoxyethyl carbamate at 300 µg/ml. These cultures constituted 3 groups, one control and two experimental, with 8 tubes in each. Two tubes from each group were harvested and counted at 12, 24, 36, and 48 hours after treatment was initiated. Cell counts obtained on each of the duplicate cultures, for a sample period, were averaged. The average number of cells for each duplicate per sample period was plotted on semilog paper (cell number vs. time). The points of the respective plots were connected to produce growth curves.

To establish doubling times from each of the growth curves (control and experimentals) lines representing multiples (powers of 2) of

the baseline number of cells were constructed horizontally on the graphs. Doubling times were extrapolated by measuring the time (abscissa) for the cell number to double (ordinant) as each curve transected the horizontal lines. Averages of the individual doubling times for each concentration and control were calculated.

Chromosome Experiments

In order to establish and compare the effects of the selected concentrations (300 and 3000 μg/ml) of the two compounds on the chromosomes of Chinese hamster fibroblasts, 25 cultures were established in plastic flasks (30 ml). The cultures were incubated 24 hours to allow the cells to settle and attach to the surfaces of the vessels. At the end of the 24-hour period, the cultures were randomly selected and divided into two groups; controls and experimentals. Five cultures were used as controls, after decantation of the 24-hour growth medium, and replenished with fresh growth medium. The experimentals contained 5 cultures for each of the concentration levels and were treated with media containing their respective compounds. All cultures were re-incubated. Two hours prior to the harvesting, all cultures were treated with 0.2 μg/ml colcemid (GIBCO). At 6, 12, 24, 48, and 72 hours after treatment was begun, one culture per treatment (control and experimental) was harvested and used to prepare slides of metaphase spreads for study. Five slides were made from each culture per sampling period. All cultures that were harvested at 72 hours received changes of their respective media at 48 hours.

The harvesting procedure for preparing metaphase spreads was as follows. The used growth media and the PSA rinses were decanted into

prelabeled 10-ml screw-cap centrifuge tubes. The cultures were treated with trypsin-EDTA and the suspensions were added to their respective centrifuge tubes. The tubes were centrifuged (as previously described) and the supernatants were discarded. To the cell pellets, a HBSS-distilled water (1:3) solution was added and the cells were resuspended. The suspended cells were heated 10 minutes in a 37°C water bath and recentrifuged. The supernatants were discarded. A 1:3 fixative (acetic acid to absolute methanol) was added gently to each tube and allowed to remain undisturbed for 30 minutes. The fixative was discarded, the cells were resuspended and centrifuged again. The latter procedure was repeated at least two times or until the suspensions became cloudy and less flocculent. With the aid of a Pasteur pipette, 5-6 drops of the suspensions were added to each of the five slides per treatment per sampling period. Prior to use, the slides were precleaned in a dichromate solution, washed in running water, and rinsed in 95% ethanol. The slides were drained briefly and stored in 20% ethanol (in a refrigerator) at 4-6°C. The above procedure, for the preparation of chromosome slides, was a modification of techniques used by: Tjio and Levan (1956); Rothfels and Siminovitch (1958); L. Higgins, Biochemistry Department, Oklahoma State University, Stillwater, Oklahoma and C. Lovig, Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma (personal communications).

The cells were stained with Giemsa (Hartman-Leddon Company, Philadelphia, Pennsylvania) for 10 minutes, rinsed with two changes of distilled water, and air-dried. The complete stain was prepared and buffered at 6.4 (Priest, 1969). Coverslips were mounted on the slides with Permount.

Ten cells with well-spread metaphase chromosomes were randomly selected under low magnification (100X) using a Zeiss Research Microscope, equipped with a 35mm camera. For each treatment and sampling period a total of 50 cells were studied. The chromosomes were scored under the oil immersion objective (1000X). The metaphase cells were studied for numerical and structural aberrations in chromosomes. Particular emphasis was placed on chromosome number, number of cells with breaks, number of breaks, types of breaks and kinds of chromosomes in which the breaks occurred. The following notations were used to denote types of chromosomes and kinds of breaks: LM (long-medium sized metacentrics or submetacentrics), ACRO (acrocentrics or subtelocentrics), SM (short metacentrics), CHR (chromatid breaks) and ISO (isochromatid breaks). Metaphase chromosomes that demonstrated a clear discontinuity in structure were recorded as having breaks. Chromosomes with faintly-stained regions were considered as having gaps but were not recorded.

Photographs of the metaphase chromosomes were made with the microscope and camera described above. All photographs were taken with Panatomic-X film (Eastman Kodak Company, Rochester, New York), processed in Kodak Microdol (1:3), and printed on Kodak Polycontrast F paper.

Reversible-Temporary or Irreversible-Permanent Effects

In an effort to ascertain whether or not the effects of the two compounds were reversible-temporary or irreversible-permanent, one concentration (300 µg/ml) for each compound was selected for study. Cells were exposed to medium containing ethyl carbamate or methoxyethyl carbamate for 36 hours (treatment period I) and cells treated

similarly were returned to culture in normal (control) medium for an additional 36-hour period (treatment period II).

In the design of this experiment 12 cultures were established in growth medium and incubated for 24 hours. At the end of 24 hours, the cultures were randomly selected and divided into 3 groups, one control and two experimentals. Each group contained four cultures. The 24-hour medium was decanted. The control cultures were treated with normal growth medium and each of the experimental groups was treated with medium containing ethyl carbamate or methoxyethyl carbamate, respectively. The cultures were reincubated for 36 hours following the onset of the treatment with or without the compounds. Two hours prior to harvesting, two cultures from each of the 3 groups were treated with 0.2 μg/ml colcemid. At the end of 36 hours, the two cultures from each group, previously treated with colcemid, were harvested and used to prepare slides for chromosome studies (4 slides/culture). The two cultures per group represented duplicates of the same treatment. All slides prepared during this investigation were made and stained in the same manner as described previously.

The respective media were decanted from the remaining six cultures (2 per group) and each culture was rinsed briefly twice with equal volumes of pre-warmed HBSS. To these cultures normal growth medium was added. The cultures were reincubated for an additional 36 hours following the treatment with their respective media. Two hours prior to harvesting, these cultures were treated with 0.2 μg/ml colcemid. At the end of the incubation period, the cultures were harvested. Slides (4 per culture) were prepared and stained as above.

From each culture at a sample period, two of the four slides were

selected randomly and well-spread metaphase cells were studied. Twenty-five cells were studied per slide. Therefore, a total of 50 metaphase cells were studied from each treatment and sample period. For each set of duplicate cultures, 100 cells were observed. The data recorded in this experiment, with reference to chromosomes, were the same as indicated in the previously mentioned study of chromosomes.

For growth measurements in this experiment, metaphase indices were used. Each metaphase index provided a ratio of the dividing cells (metaphases) to non-dividing cells per thousand. To obtain these data, the same four slides (per culture) from the above chromosome studies were used. From each slide 250 cells were randomly counted using high-dry optics to give a total of 1000 cells. The cells were scored as metaphases or non-metaphases.

For analyses, in this experiment, comparisons were made between chromosome data and metaphase indices within the two sample periods for each treatment. Comparisons were also made between chromosome data between sample periods 1 (first 36 hours) and 2 (second 36 hours). No comparisons of the metaphase indices were made between the two sample periods.

Statistical Analyses

Chi-Square (X^2) Test:

For experiments in which chromosome numbers and number of cells with breaks were studied, the Chi-square (X^2) test was usually employed, except in one case. This exception is discussed under a subsequent heading, analysis of variance. For X^2 analyses of the chromosome number data, the metaphase cells were distributed into 5 classes or

groups. The groupings followed a modification of a classification used by Ford and Yerganian (1958). The five groups of metaphases were: I, less than "diploid" (<20 chromosomes); II, approximately "diploid" (20-25 chromosomes); III, less than "triploid and tetraploid" (26-29 chromosomes); IV, "triploid and tetraploid" (30-50 chromosomes); and V, more than "tetraploid" (>50 chromosomes).

In the computation of each X^2 value, the distribution of the chromosome numbers of the control, for a sample period, was used as the expected distribution. The distribution of chromosome numbers for each of the experimentals was used as the observed distribution and tested against its respective control. With 5 classes or groups 4 (n-1) degrees of freedom were used.

For analyses involving data on the number of cells with breaks, two classes or groups were established. The groupings were: the number of cells with breaks and the number of cells without breaks. For the two classes, one (n-1) degree of freedom was used. Similar to the above procedure, the number of control cells with and without breaks were used as the expected distributions. The number of experimental cells with and without breaks were used as the observed distributions and tested against the untreated control distribution for a given sample period.

For the computations of the data on number and breakage, the null hypothesis of no difference between treatments (controls vs. experiments) was assumed. The significance level chosen was P=0.050.

Analysis of Variance, F-test, and Least Significant Differences:

Analysis of variance was used to analyze the data from the growth

rate experiments for each compound. The growth ratio data from the 4 experiments (A, B, C and D) involving ethyl carbamate were pooled and analyzed by this method. The three experiments (E, F and G) for methoxyethyl carbamate were analyzed similarly. In both sets of experiments, F-tests were done to test the null hypothesis of no differences of effects between the various concentration levels of the compounds.

To test for differences between the means of the growth ratios for each dosage level, the least significant difference (LSD) method was employed. In other words if the difference between a pair of means was greater than the calculated LSD value, the effects of the concentrations on the growth rate of the cultured cells were statistically significant.

For statistical treatment of the experimental data on doubling time, analysis of variance, F-test and LSD were used.

For the experiment in which ethyl carbamate and methoxyethyl carbamate (300 μg/ml) were tested and compared with reference to effects classified as reversible-temporary or irreversible-permanent, data on chromosome breakage were analyzed by analysis of variance. In the same experiment, growth rate data as assessed by metaphase indices were also analyzed by this procedure. Chromosome numbers for this experiment were analyzed from pooled sampled (duplicates of the same treatment per sample period) by the statistical test above (X^2).

To compare the effects of the compounds on chromosome breakage within treatment period I (36 hours of exposure to the compounds), the hypothesis (Ho: $\mu_C=\mu_E=\mu_M$) was tested by an F-test. The symbols μ_C, μ_E, and μ_M represent the means of the controls, ethyl carbamate

and methoxyethyl carbamate, respectively. The same hypothesis and test were used to test the effects of the compounds on chromosome breakage after cells similarly treated were returned to normal medium for an additional 36 hours (treatment period II). The analysis of variance and F-test, in these two cases, were used to compare differences between treatments at a fixed time.

Analysis of variance and F-tests were used to compare times (treatment periods) for a fixed treatment. In other words each treatment was tested against itself between the two periods (I and II). The hypotheses tested were: Ho: $\mu_C I = \mu_C II$, $\mu_E I = \mu_E II$ and $\mu_M I = \mu_M II$ for controls, ethyl carbamate and methoxyethyl carbatate, respectively.

The data on metaphase indices were analyzed by analysis of variance and F-tests. Only differences between treatments at a fixed time were calculated.

t - Tests:

To test for significance between treatment means within and between the two sample periods, from breakage data, the t- test was used. For data on metaphase index, the t - test was used only to test for significance of the effects within the two sample periods.

In all cases in which F and t - test were used, P = 0.050 was chosen as the significance level.

CHAPTER III

RESULTS

Growth Rate Experiments

In order to ascertain and compare the effects of ethyl carbamate and methoxyethyl carbamate and to select concentrations of these compounds for further studies, several experiments were conducted. In these initial experiments, Chinese hamster fibroblasts were exposed for 72 hours to media containing 0, 30, 300, 3000, and 30,000 μg/ml for each of the compounds. Four separate experiments (A, B, C and D) using these dosage levels of ethyl carbamate were conducted and three experiments (E, F and G) for methoxyethyl carbamate. The results of each experiment, at each dosage level were expressed as growth ratios. The ratios for experiments with ethyl carbamate are shown in Table I.

As shown in Table I, and based on the averages of the 4 experiments, concentrations of 30 and 300 μg/ml demonstrated growth ratios of 1.24 and 1.10, respectively. The results indicated stimulatory effects. The growth ratio for 30 μg/ml was statistically significant, but not significant for 300 μg/ml. At concentrations of 3000 and 30,000 μg/ml the averages of the ratios were 0.92 and -0.06 respectively. Although treatment with ethyl carbamate at 3000 μg/ml was not statistically different from the control, this concentration showed a trend of slight retardation of cell growth. Statistical significance was shown for 30,000 μg/ml and this level was considered lethal.

TABLE I

EFFECTS OF A 72-HOUR EXPOSURE TO ETHYL CARBAMATE ON THE GROWTH RATE OF CHINESE HAMSTER FIBROBLASTS

Concentrations (μg/ml)	Growth Ratios for Experiments					S.E.
	A	B	C	D	MEAN	
0	1.0000	1.0000	1.0000	1.0000	1.00	0.00
30	1.3152	1.3487	1.1661	1.1395	1.24	0.05
300	1.0066	1.3965	0.7892	1.1888	1.10	0.13
3000	0.9860	1.0368	0.9410	0.7016	0.92	0.07
30000	-0.0521	-0.0583	-0.0676	-0.0528	-0.06	0.01

Note: The five digits for the growth ratios are not significant but were used in the computation of the means and standard errors.

Analysis of variance and an F-test based on these data showed that highly significant statistical differences existed between the various dosages (Table II). Analysis by the LSD procedure revealed that the growth ratios for concentrations of 30 and 30,000 μg/ml were statistically different from the controls. No statistical difference existed between the growth ratios of 30 and 300 μg/ml, but there were statistical differences between the growth ratios for 30, 3000 and 30,000 μg/ml. No statistical difference existed between the growth ratios for 300 and 3000 μg/ml.

Microscopic observations, at the end of 72 hours, revealed that cells treated with media containing ethyl carbamate in concentrations of 0, 30, 300 and 3000 μg/ml were firmly attached to the surfaces of

their respective flasks and forming sheet-like monolayers. The cells were flattened and showed clear detail. The indicator in the media for the above concentrations, in most experiments, had a yellowish color. Cells which were exposed to 30,000 μg/ml of the compound showed a loss of adherence to the flasks. The few cells which remained attached were rounded, highly granular and cytolytic. These cells were considered deceased. The medium in which the latter cells were cultured contained a great deal of debris. The pH of these cultures remained basically the same as upon initial inoculation or appeared slightly alkaline.

TABLE II

ANALYSIS OF VARIANCE FOR EXPERIMENTS A, B, C AND D

Source of Variation	df	SS	MS	F_{cal}
Total	19	3.7722		
Between Treatments (Concentrations in Experiments A,B,C and D)	4	3.4722	0.8680	43.4
Within Treatments (Concentrations in Experiments A,B,C and D)	15	0.3000	0.0200	

$F\ tab_{.05(4,15)} = 2.58$ LSD = 0.2131

The growth ratios at the various concentrations of methoxyethyl

carbamate are shown in Table III. The averages of the growth ratios for methoxyethyl carbamate in an increasing level of concentration were 1.41, 1.36, 2.36 and -0.21, respectively. According to the data presented in the table, methoxyethyl carbamate in dosages of 30, 300 and 3000 μg/ml showed statistically significant stimulatory effects when compared with the control, while 30,000 μg/ml demonstrated statistically significant effects which were considered lethal. The statistical significance of these effects were computed by analysis of variance, F-test and LSD procedures (Table IV). The LSD method for comparison of the difference between the growth ratios at 30 and 300 μg/ml showed no significant difference between these two drug levels. Although these two drug levels were not statistically different from each other, both were statistically different from all other comparisons of the mean growth ratio.

TABLE III

EFFECTS OF A 72-HOUR EXPOSURE TO METHOXYETHYL CARBAMATE ON THE GROWTH RATE OF CHINESE HAMSTER FIBROBLASTS

Concentrations (μg/ml)	Growth Ratios for Experiments				S.E.
	A	B	C	MEAN	
0	1.0000	1.0000	1.0000	1.00	0.00
30	1.5056	1.4082	1.3271	1.41	0.05
300	1.3078	1.3930	1.3766	1.36	0.03
3000	2.2829	2.4035	2.2935	2.36	0.03
30000	-0.2435	-0.1078	-0.2895	-0.21	0.05

TABLE IV

ANALYSIS OF VARIANCE FOR EXPERIMENTS E, F AND G

Source of Variation	df	SS	MS	F_{cal}
Total	14	10.4074		
Between Treatments (Concentrations in Experiments E, F and G)	4	10.3627	2.5906	588.77
Within Treatments (Concentrations in Experiments E, F and G)	10	0.0447	0.0044	

$F\ tab_{.05(4,10)} = 3.48$ LSD = 0.1152

All of the cultures in these experiments were also observed microscopically at the end of 72 hours. Cell cultures treated with or without media containing methoxyethyl carbamate in concentrations of 0, 30, 300, and 3000 µg/ml showed cells firmly attached and formed sheet-like monolayers. These cells showed clear cellular detail and were flattened. The pH of these cultures, on the average, was indicative of active metabolism and showed a yellowish tinge. Cell cultures exposed to methoxyethyl carbamate at 30,000 µg/ml showed microscopic and pH characteristics as previously described for ethyl carbamate at this dosage level.

For comparative purposes, the averages of the growth ratios for the 4 experiments using ethyl carbamate and the 3 experiments with

methoxyethyl carbamate are graphically illustrated in Figure 1. Also shown in the figure, by lines parallel to the Y axis, are standard errors of the growth ratios from the experiments per dosage level. As shown in the figure, methoxyethyl carbamate was a more effective stimulator of growth than was ethyl carbamate. Both compounds showed stimulatory effects at 30 and 300 μg/ml. At 3000 μg/ml the effects of the two compounds differed; ethyl carbamate tended to show a trend towards retardation, while methoxyethyl carbamate induced an acceleration of growth. Both of these compounds at 30,000 μg/ml were lethal.

In an effort to establish the effects of dosages between 3000 and 30,000 μg/ml for both of the test compounds, concentrations of 6000, 9000, 12,000 and 15,000 μg/ml were tested in two different experiments (one for each compound). Experiment H was designated for ethyl carbamate and Experiment I was assigned to testing the effects of methoxyethyl carbamate.

For comparisons of the results, the growth ratios for the two experiments are plotted and shown in Figure 2. Based on the results illustrated in the figure, ethyl carbamate showed an increasing trend of retardation of cell growth at concentrations of 6000 and 9000 μg/ml when compared with the control. The growth ratios for these two concentrations were 0.79 and 0.40, respectively. Concentration levels of 12,000 and 15,000 μg/ml, for the compound, showed a pattern of increasing lethality with growth ratios of -0.02 and -0.08, respectively. Ethyl carbamate at 12,000 μg/ml showed 15.88% lethality with 49.78% lethality at 15,000 μg/ml.

Microscopic observations of the cultures in Experiment H revealed that cells treated with media with or without ethyl carbamate in

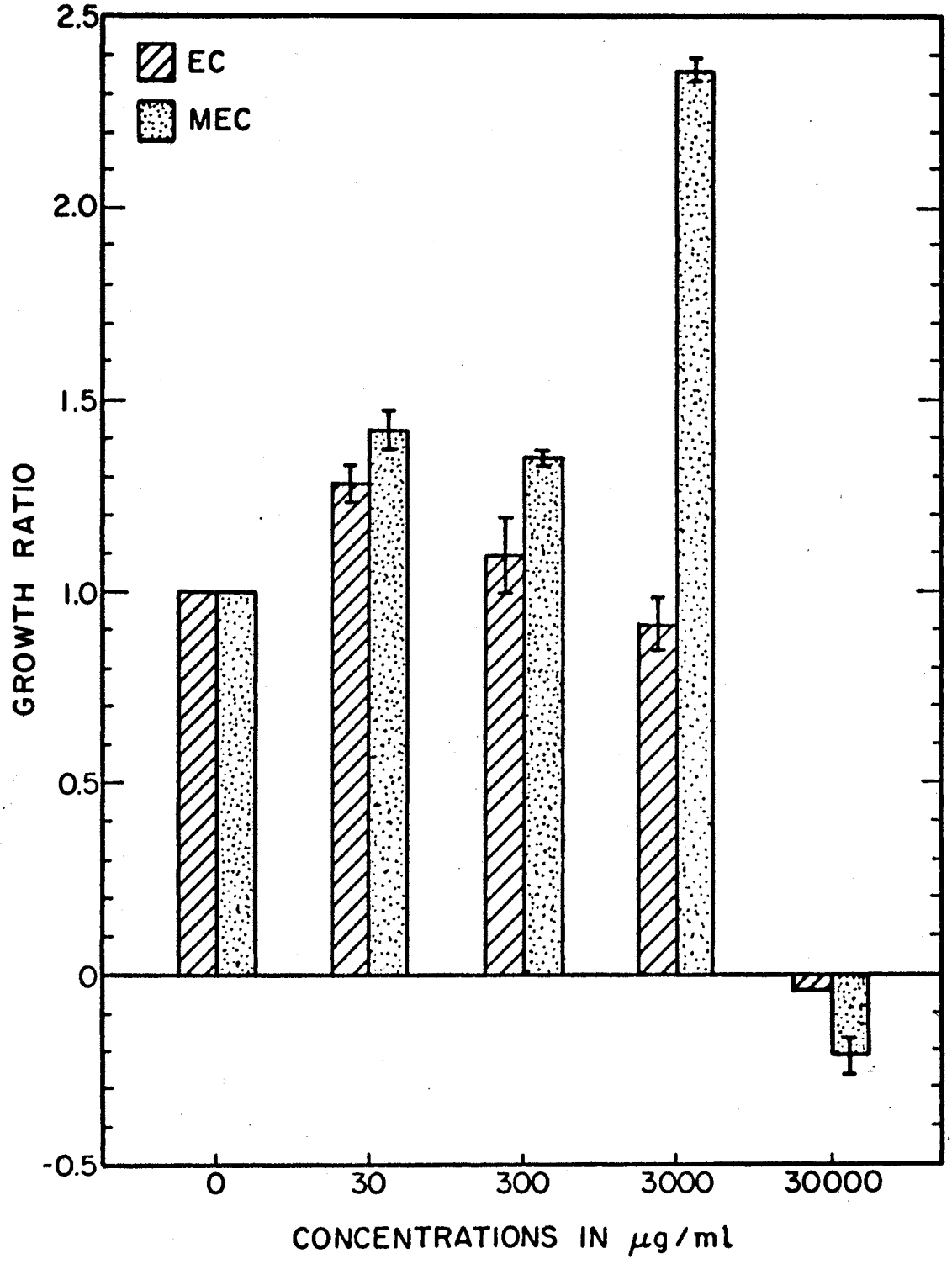


Figure 1. A Comparison of the Mean Effects of a 72-hour Exposure to Various Concentrations of Ethyl Carbamate and Methoxyethyl Carbamate on the Growth Rate of Chinese Hamster Fibroblasts.

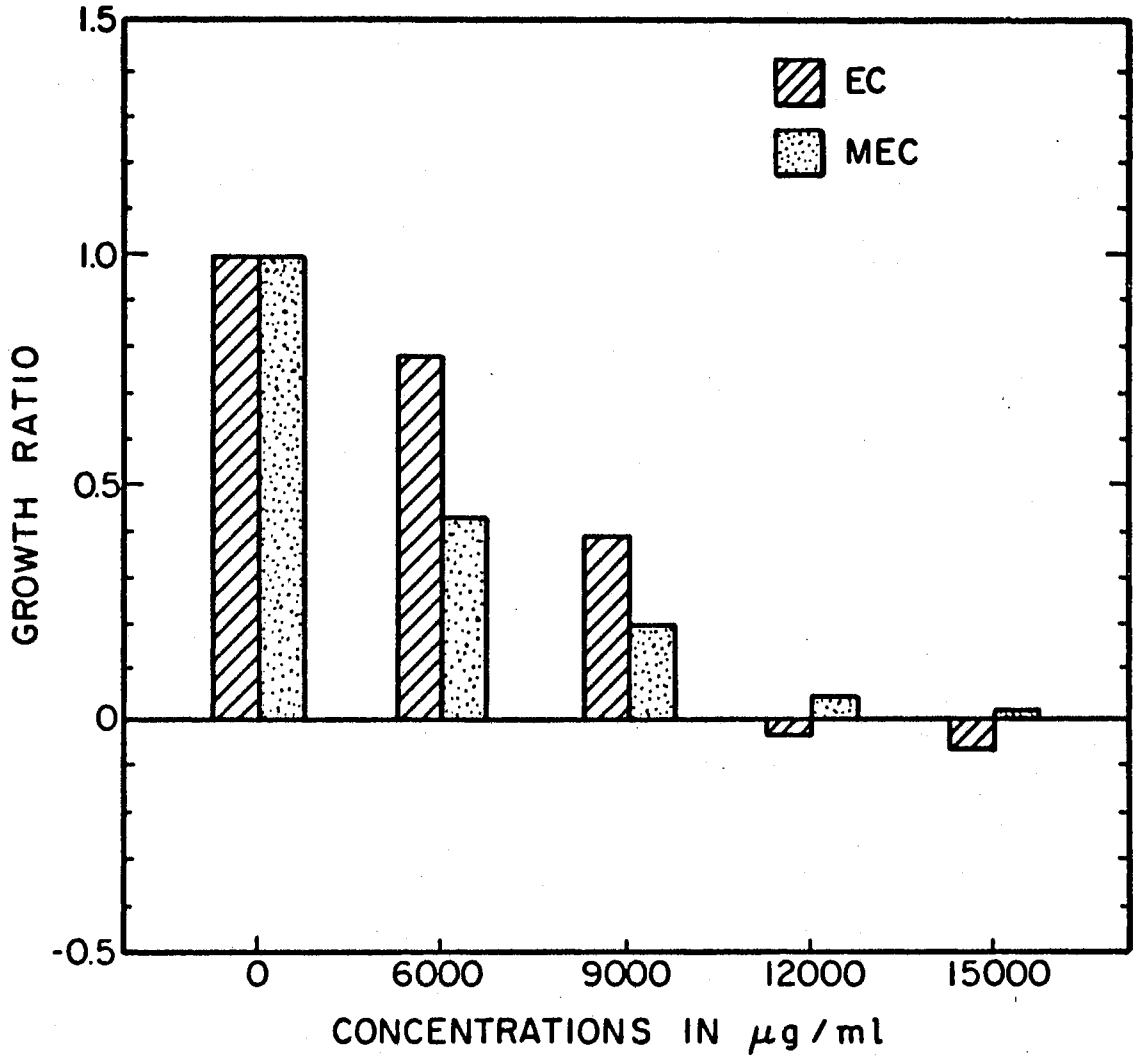


Figure 2. A Comparison of the Effects of a 72-hour Exposure to Various Concentrations of Ethyl Carbamate and Methoxyethyl Carbamate on the Growth Rate of Chinese Hamster Fibroblasts.

dosages of 0 and 6000 µg/ml were firmly attached to their respective flasks and formed a confluent to near-confluent monolayer. Cells were flattened and showed clear detail. The respective media were yellow in color indicating that active proliferation of cells occurred. Cells exposed to media containing 9000 µg/ml showed mostly flattened and healthy cells. However, some of the observed cells showed granulation. The cells exposed to this concentration were less numerous as compared to the controls. The color of the medium was a yellowish-orange. Cells exposed to ethyl carbamate at 12,000 µg/ml were mostly flattened, but sparse. Some cells in these cultures were rounded with granulation and had become detached from the surfaces of the cultures. The color of the medium was a reddish-orange. At 15,000 µg/ml poor growth was evident from microscopic observations. Many rounded cells with a loss of detail were observed. The medium contained a number of floating cells and cellular debris. The color of the medium appeared to retain its original color which indicated some interference with normal acid production and metabolism was evident.

The results of Experiment I, as shown in Figure 2, revealed that methoxyethyl carbamate showed a direct proportional relationship with cell growth. As the concentration of the compound increased retardation of cell growth increased. The growth ratios for the compound were 0.44, 0.20, 0.05, 0.02 for dosages of 6000, 9000, 12,000 and 15,000 µg/ml, respectively. Although these dosages levels showed retardation effects none were considered lethal.

Microscopic observations of the cultures at the end of 72 hours for this experiment revealed that cells treated with media with or without methoxyethyl carbamate in dosages of 0 and 6000 µg/ml, were

firmly attached to their respective flasks. The cells formed a confluent and near-confluent monolayer for these two treatments respectively. The cells were flattened and showed clear detail. At 9000 µg/ml, cell density was sparse when compared to controls, but cells were flattened and appeared healthy. At a concentration of 12,000 µg/ml methoxyethyl carbamate most cells, again, were flattened, well attached, but less numerous when compared with control cultures. The medium in which these cells were cultured did, however, contain some floating cells. At 15,000 µg/ml, cell density was very sparse. Some cells appeared well attached and healthy, while others appeared to have become detached from the surfaces of the flasks and floated in the medium. Some rounded and granular cells were also observed. The pH (as determined by changes in the color of phenol red) ranged from yellow to pinkish-orange. Control and medium containing methoxyethyl carbamate at 6000 µg/ml appeared yellow. Colors of orange to pinkish-orange were observed for media containing 9000, 12,000 and 15,000 µg/ml, respectively.

Based on the results of both of these groups of experiments (A, B, C, D and H) for ethyl carbamate and (E, F, G and I) for methoxyethyl carbamate, the author selected the concentration levels of 300 and 3000 µg/ml for both compounds to be used in further studies. Both of these levels for the two compounds were considered sublethal but showed effects on the growth rates of the cultured cells.

Doubling Time Experiments

In order to establish and compare the effects of the selected concentrations (300 and 3000 µg/ml) for each test compound on the

doubling time of cell populations, two separate experiments were conducted. In each experiment, identical concentration levels of the two compounds were used. Cell populations, from duplicate cultures of the same treatment, were enumerated every 12 hours over a 48-hour period. A summary of the results of the experiment in which 300 μg/ml of each compound was used is shown in Table V.

TABLE V

EFFECTS OF ETHYL CARBAMATE AND METHOXYETHYL CARBAMATE AT CONCENTRATIONS OF 300 μg/ml ON THE DOUBLING TIME OF CHINESE HAMSTER FIBROBLASTS

Doubling Periods	Treatments and Doubling Times in Hours		
	Control	EC	MEC
I	16.5	12.0	13.5
II	6.5	9.0	10.5
III	7.2	10.0	11.2
IV	7.3	11.2	9.0
TOTAL	37.5	42.2	44.7
Mean	9.4	10.5	11.2
S.E.	2.38	0.66	0.94
$LSD_{.05}$	4.54		

In the table, the individual and average doubling times (in hours)

for each treatment (control and experimentals) are presented. Individual doubling times of the control populations were 16.5, 6.5, 7.2 and 7.3 hours respectively for the four periods (I, II, III and IV). The average doubling time and standard error of this estimate were 9.4 hours and 2.38, respectively. Individual doubling times of the cell populations treated with ethyl carbamate were 12.0, 9.0, 10.0 and 11.2 hours. The average time required for cell populations to double with this treatment was 10.5 hours with a standard error of 0.66. For populations of cells treated with medium containing methoxyethyl carbamate, the individual doubling times were 13.5, 10.5, 11.2 and 9.0 hours, respectively. The average doubling time and standard error were 11.2 hours and 0.94, respectively.

Based on the average times required for the cell populations to double in this experiment, differences were noted. The control treatment reduced the amount of time required for the cell populations to double by 1.1 and 1.8 hours when compared with the effects of treatments of ethyl carbamate and methoxyethyl carbamate, respectively. However, analysis of variance and an F-test revealed that these differences between the populations (control and experimentals) were not statistically significant at the 0.05 level (Table VI).

The results of this experiment are also depicted in a graphic form in Figure 3. The growth curves were plotted as cell number against time from the averaged data taken at each sample period for each treatment. According to the data presented, the growth of the control populations delayed or lagged for the first 12 hours, while each of the experimental populations within the same period showed accelerated growth. The overall results of the experiment showed that populations

of cells treated with control medium were in a lag phase, in the first 12 hours in exponential growth between 12 and 36 hours, and tended to move towards a stationary phase between 36 and 48 hours. Cell populations treated with experimental media (ethyl carbamate or methoxyethyl carbamate) remained in exponential growth phases from time 0 to time 48 hours. The growth curves showed clearly that all populations doubled slightly more than 4 times. Also shown is the fact that the first doubling time for all populations was the longest with all others reduced.

TABLE VI

ANALYSIS OF VARIANCE FOR THE EFFECTS OF ETHYL CARBAMATE AND METHOXYETHYL CARBAMATE (300 μg/ml) ON THE DOUBLING TIME OF CHINESE HAMSTER FIBROBLASTS

Source of Variation	df	SS	MS	F_{cal}
Total	11	89.7425		
Between Treatments	2	17.0275	8.5137	1.05475
Within Treatments	9	72.7250	8.07944	

$F_{tab\ .05\ (2,9)} = 4.26$

Results of the second experiment in which 3000 μg/ml of each of the experimental compounds were used are summarized in Table VII. The table shows the individual and average doubling times (in hours) for each treatment. Cell populations treated with medium containing

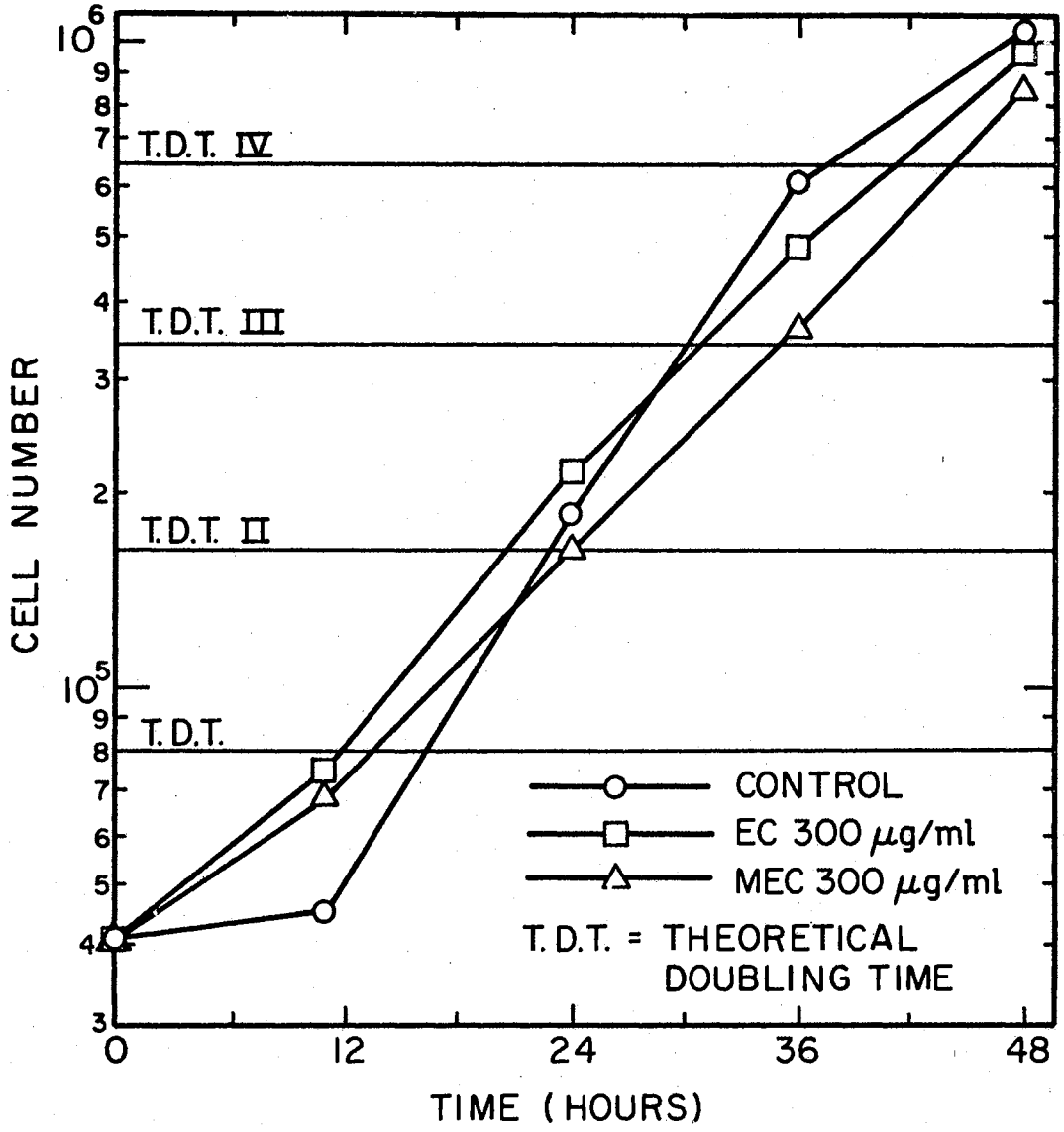


Figure 3. A Comparison of the Effects of Ethyl Carbamate and Methoxyethyl Carbamate (300 μg/ml) on the Doubling Time of Chinese Hamster Fibroblasts.

methoxyethyl carbamate doubled nearly 4 times in 48 hours, while the control and ethyl carbamate treated populations doubled nearly 3 and $2\frac{1}{2}$ times, respectively. For equal comparison of the treatments only two of the doubling times are presented in the table for each treatment. The individual doubling times of cell populations for the two periods (I and II), treated with control, ethyl carbamate or methoxyethyl carbamate medium were 16.0, 13.5; 13.5, 19.5; 17.0 and 10.5, respectively. The average time required for cell populations to double when treated with control medium was 14.7 hours with a standard error of 1.25. The same for populations treated with ethyl carbamate was 16.5 hours and a standard error of 3.00. For cell populations treated with methoxyethyl carbamate, the average time required for doubling was 13.7 hours with a standard error of 3.25.

TABLE VII

EFFECTS OF ETHYL CARBAMATE AND METHOXYETHYL CARBAMATE AT CONCENTRATIONS OF 3000 μg/ml ON THE DOUBLING TIME OF CHINESE HAMSTER FIBROBLASTS

Doubling Periods	Treatments and Doubling Times in Hours		
	Control	EC	MEC
I	16.0	13.5	17.0
II	13.5	19.5	10.5
Total	29.5	33.0	27.5
Mean	14.7	16.5	13.7
SE	1.25	3.00	3.25
$LSD_{.05}$	10.39		

Based on the averages of the doubling times, it appears that methoxyethyl carbamate caused a reduction in the amount of time required for the cell populations to double when compared with the effects produced by ethyl carbamate or control treatment. There were differences of 1.0 and 2.8 hours required for doubling for the effects of ethyl carbamate and control treatments, respectively, as compared with treatment with methoxyethyl carbamate. Control populations showed a trend of doubling that exceeded the doubling rate of cultures treated with ethyl carbamate. However, analysis of variance and an F-test revealed that no statistically significant differences existed between the various treatments (Table VIII).

TABLE VIII

ANALYSIS OF VARIANCE FOR THE EFFECTS OF ETHYL CARBAMATE AND METHOXYETHYL CARBAMATE (3000 μg/ml) ON THE DOUBLING TIME OF CHINESE HAMSTER FIBROBLASTS

Source of Variation	df	SS	MS	F_{cal}
Total	5	54.7083		
Between Treatments	2	22.7083	11.3541	1.0644
Within Treatments	3	32.0000	10.6666	

$F\ tab_{.05\ (2,3)} = 9.55$

The results of this experiment are graphically illustrated in

Figure 4. The control populations showed approximately exponential growth throughout the entire experiment. Populations of cells treated with methoxyethyl carbamate showed the same trend, but the slope of the curve indicated a much more accelerated growth. The overall pattern of growth for populations of cells treated with ethyl carbamate showed a diauxic (double) or bimodal trend of growth. The growth curve for treatment with this compound showed accelerated slopes between times 0 and 12 hours and 24 and 36 hours. During the periods of 12-24 hours and 36-48 hours growth of the populations appeared retarded.

The Effects of Ethyl Carbamate and Methoxyethyl Carbamate on the Chromosomes of Chinese Hamster Fibroblasts

In order to determine and compare the effects of ethyl carbamate (EC) and methoxyethyl carbamate (MEC) on the chromosomes of Chinese hamster fibroblasts, two sublethal concentrations (300 and 3000 μg/ml) of the compounds were used. Cells were exposed to media containing these dosages of the respective compounds for 6, 12, 24, 48 and 72 hours. Control cells were similarly exposed to medium without either of the compounds. Slides for each of the sample periods were prepared and studied for structural and numerical aberrations of the metaphase chromosomes. With reference to structural aberrations, major emphasis was placed on the study of chromosomal breaks. For each sample period, each experimental treatment (ethyl carbamate or methoxyethyl carbamate at 300 or 3000 μg/ml) was compared with its control. The results presented in this section come under two main headings: effects on chromosomal breakage and effects on chromosome number.

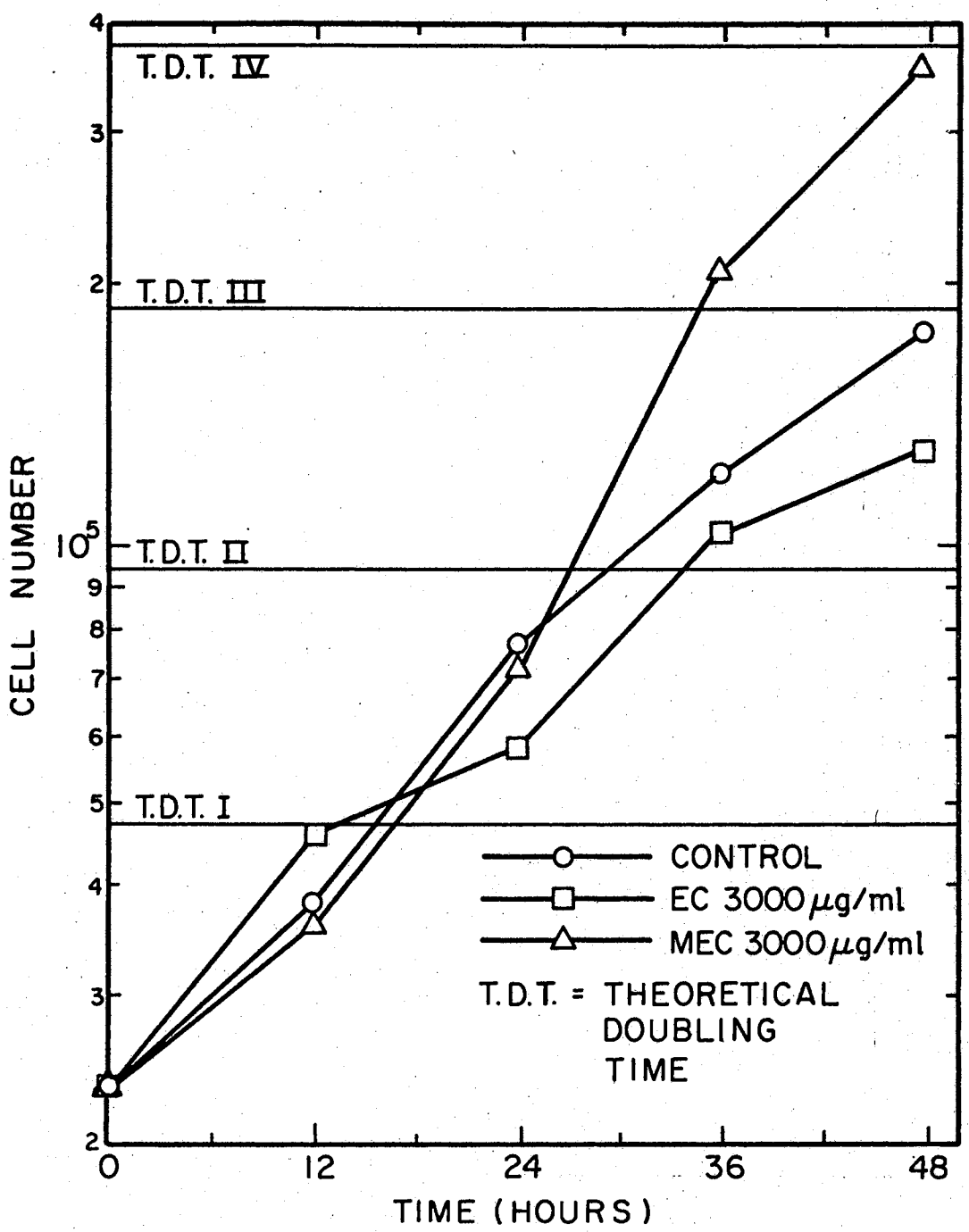


Figure 4. A Comparison of the Effects of Ethyl Carbamate and Methoxyethyl Carbamate (3000 μg/ml) on the Doubling Time of Chinese Hamster Fibroblasts.

Effects on Chromosomal Breakage

The effects of the experimental and control treatments on chromosomal breakage are summarized in Table IX. These relationships are more clearly depicted in Figure 5 where the number of cells with chromosome breaks per 50 cells are plotted as a function of length of time of exposure. The results of the 6-hour exposure to ethyl carbamate 300 and 3000, and methoxyethyl carbamate at 3000 μg/ml showed statistically significant numbers of cells with breaks. Methoxyethyl carbamate at 300 μg/ml demonstrated more cells with breaks when compared with the control, but the difference was not statistically significant. For the 12-hour exposure period, the results were the same as those discussed for the 6-hour exposure period. However, methoxyethyl carbamate at 300 μg/ml showed the same number of cells with breaks as the control cells. At the 24-hour exposure period, all experimental treatments showed statistically significant numbers of cells with breaks when compared with the number of breaks for the control cells. Also for this period, cells exposed to ethyl carbamate and methoxyethyl carbamate at 300 μg/ml demonstrated a higher frequency of breaks, than in the previous two periods. The numbers of cells with breaks were 14/50 and 16/50 respectively. After 48 hours of exposure, all experimental concentrations showed statistically significant numbers of cells with breaks when compared with the 48-hour control. At 300 μg/ml of methoxyethyl carbamate, the number of cells with breaks reached a peak with 49/50 that showed structural damage of the chromosomes. Many of the cells which demonstrated breaks at this sample period and concentration had multiple breaks,

TABLE IX

EFFECTS OF ETHYL CARBAMATE AND METHOXYETHYL CARBAMATE AT 300 AND 3000 μg/ml ON THE NUMBER OF CHROMOME BREAKS PER FIFTY CELLS AFTER VARIOUS EXPOSURE PERIODS

Length of Exposure (Hours)	Concentrations (μg/ml)	Cells With Breaks (No./50 Cells)	(%)	X^2cal	P
	0.00	3	6		
	EC 300	11	22	22.6950	<0.005
6	EC 3000	12	24	28.7234	<0.005
	MEC 300	5	10	1.4184	<0.250
	MEC 3000	8	16	8.8652	<0.005
	0.00	4	8		
	EC 300	8	16	4.0870	<0.050
12	EC 3000	9	18	6.7935	<0.010
	MEC 300	4	8	0.0000	<0.995
	MEC 3000	9	18	6.7935	<0.010
	0.00	4	8		
	EC 300	14	28	27.1739	<0.005
24	EC 3000	10	20	9.7826	<0.005
	MEC 300	16	32	39.1304	<0.005
	MEC 3000	9	18	6.7935	<0.010
	0.00	5	10		
	EC 300	11	22	8.0000	<0.005
48	EC 3000	10	20	5.5555	<0.025
	MEC 300	49	98	430.2222	<0.005
	MEC 3000	12	24	10.8889	<0.005
	0.00	2	4		
	EC 300	6	12	8.3333	<0.005
72	EC 3000	8	16	18.7500	<0.005
	MEC 300	7	14	13.0208	<0.005
	MEC 3000	7	14	13.0208	<0.005

$X^2_{tab.05(1d.f.)}=3.84$

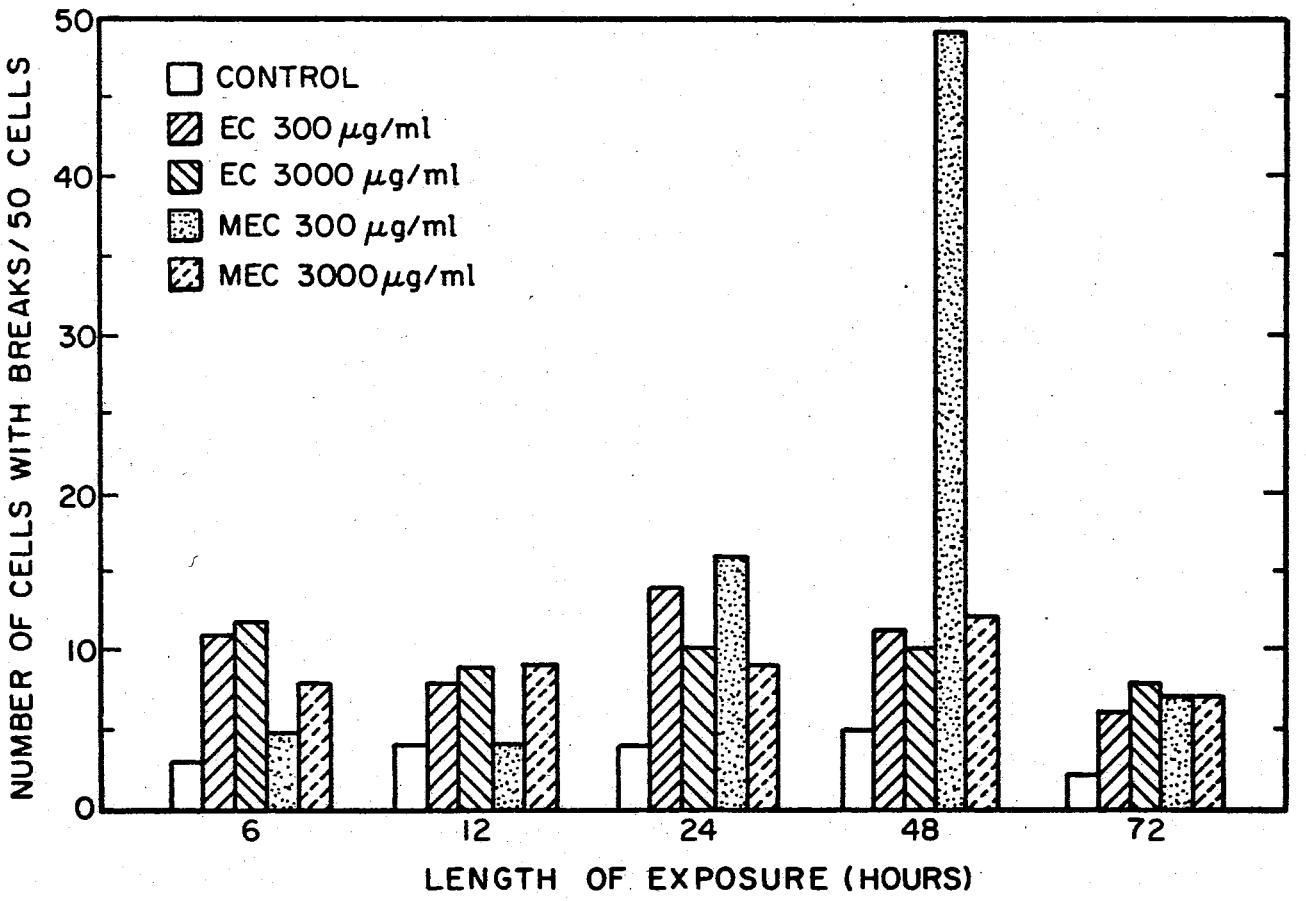


Figure 5. Effects of Ethyl Carbamate and Methoxyethyl Carbamate at Concentrations of 300 and 3000 μg/ml on Chromosome Breakage After Various Periods of Exposure.

pulverized or highly fragmented chromosomes, Figures 6, 7 and 8. Results of the 72-hour exposure to the compounds showed that statistically significant numbers of the cells had chromosomal breaks. The effects of ethyl carbamate at 300 μg/ml remained rather consistent throughout the experiment except for the peak at 24 hours and the decline after 72 hours of exposure. Ethyl carbamate at 3000 μg/ml also showed a somewhat consistent trend in the number of cells with chromosomal breaks with its maximum effects exerted after 6 hours of exposure. The effects of methoxyethyl carbamate at 300 μg/ml on the number of cells with breaks increased with time to 48 hours, then declined sharply after 72 hours. At 3000 μg/ml of methoxyethyl carbamate, the number of cells with breaks remained stable except for a peak at 48 hours.

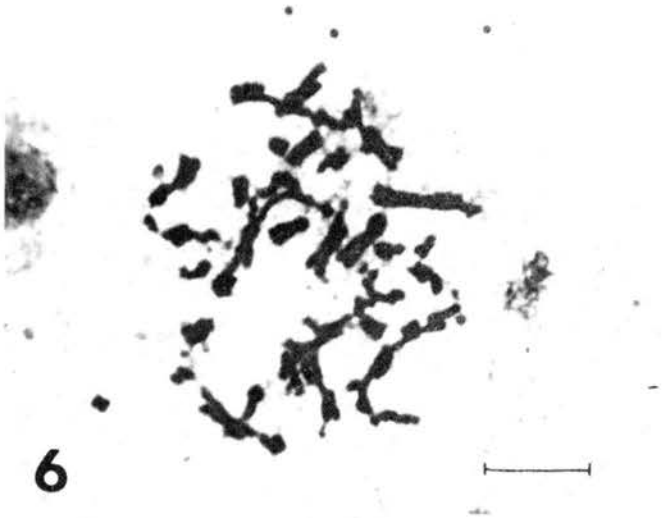
The overall results of this experiment, with reference to chromosome breakage, were obtained by pooling the data for each concentration at each exposure period and are presented in Table X. The results given in the table disregard the effects of the compounds on the cells at individual exposure periods. In the absence of either of the two test compounds and their respective concentration levels, only 7.2% of the cells demonstrated chromosomal breaks. At 300 μg/ml of ethyl carbamate, the percentage of the cells with breaks was 20.0%. When the effects of this treatment were tested against the control ratio, a high statistical significance was obtained. Similarly, the percentage of cells with breaks for ethyl carbamate at 3000 μg/ml was 19.6 and was highly significant when compared with the control. For methoxyethyl carbamate at 300 and 3000 μg/ml, the percentages of cells with breaks were 32.4% and 18.0%, respectively, with both highly sig-

Figure 6. Photomicrograph of a Chinese Hamster Metaphase Chromosome Spread Showing Multiple Breaks and Fragmentation of the Chromosomes after 48 Hours of Exposure to Methoxyethyl Carbamate (300 µg/ml).

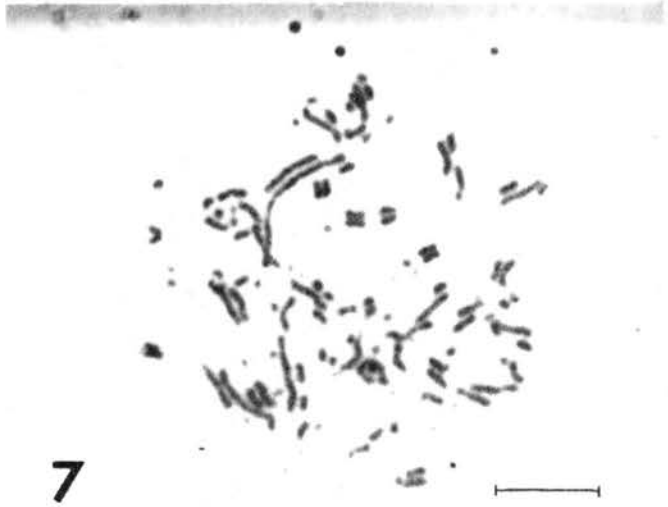
Figure 7. Photomicrograph of a Chinese Hamster Metaphase Chromosome Spread Showing Highly Fragmented Chromosomes After 48 Hours of Exposure to Methoxyethyl Carbamate (300 µg/ml).

Figure 8. Photomicrograph of a Chinese Hamster Metaphase Chromosome Spread Showing Completely Fragmented or Pulverized Chromosomes After 48 Hours of Exposure to Methoxyethyl Carbamate (300 µg/ml).

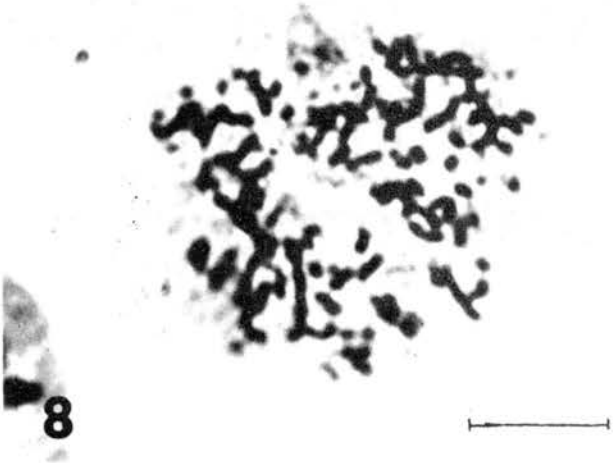
Bars Represent 10 Microns.



6



7



8

nificant. According to the results of this experiment, exposures to methoxyethyl carbamate at 300 μg/ml produced the greatest number of cells with chromosomal breaks on an overall basis.

TABLE X

SUMMARY OF THE OVERALL EFFECTS OF ETHYL CARBAMATE METHOXYETHYL CARBAMATE ON CHROMOSOME BREAKAGE

Length of Exposure (Hours)	Concentrations (μg/ml)	Cells with Breaks (No./250 cells)	(%)	X^2_{cal}	P
0-72	0.00	18	7.2		
0-72	EC 300	50	20.0	61.3027	<0.005
0-72	EC 3000	49	19.6	57.5311	<0.005
0-72	MEC 300	81	32.4	237.6078	< 0.005
0-72	MEC 3000	45	18.0	43.6422	<0.005

$X^2_{tab.05\ (1\ d.f.)}=3.84$

A summary of the number of breaks, types of breaks and kinds of chromosomes in which the breaks occurred is presented (Table XI) for each treatment and exposure period. For convenience in the study of the types of chromosomes with breaks, the chromosomes were grouped as long metacentric (LM), acrocentric or subtelocentric (ACRO) and short-metacentric (SM). An example of a normal karyotype presenting this classification is shown in Figure 9. For all of the cells with breaks studied, the breaks were of two types, chromatid (CHR) and

TABLE XI

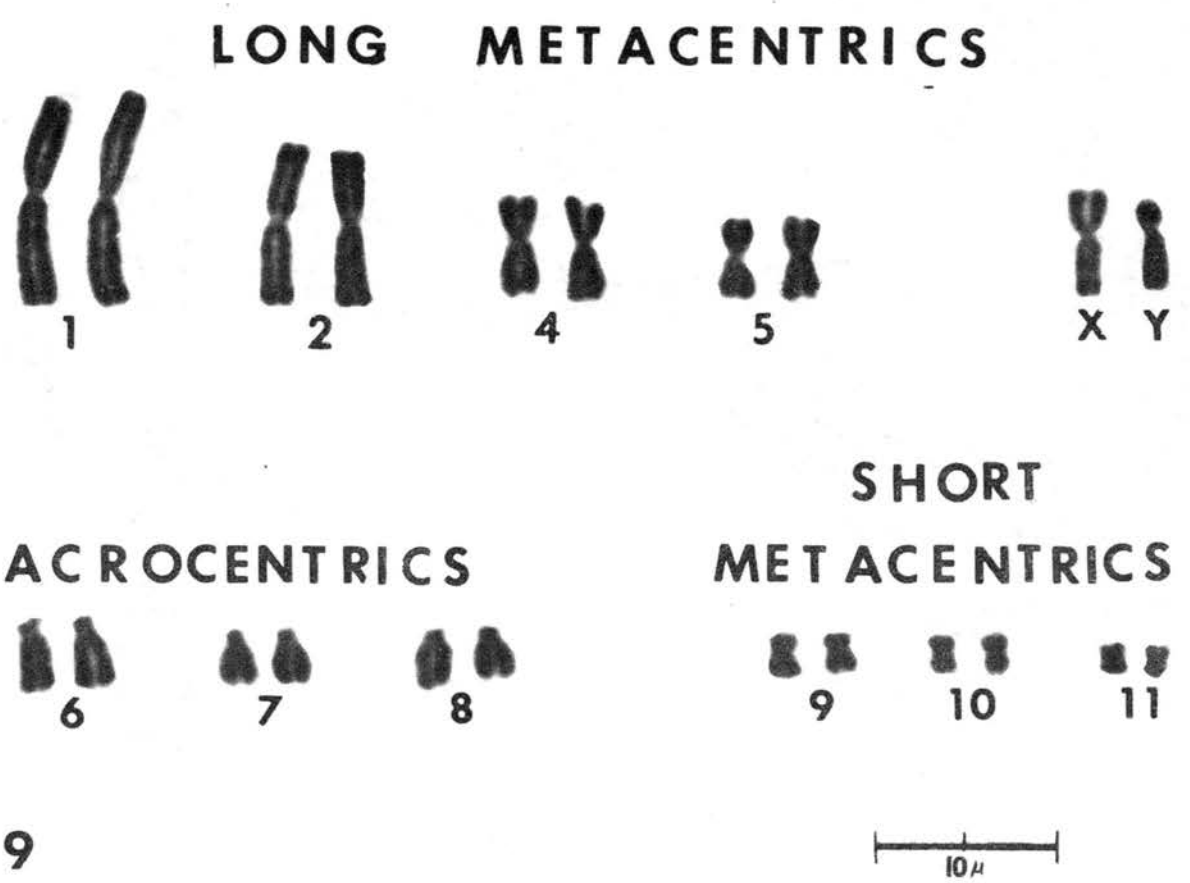
DISTRIBUTION OF THE TYPES OF CHROMOSOMES BREAKS AND KINDS OF CHROMOSOMES AFFECTED

Length of Exposure (Hours)	Concentrations (µg/ml)	Cells With Breaks (No/50 Cells)	Breaks (No.)	Chromosomes Affected and Types of Breaks												Total CHR Breaks		Total ISO Breaks	
				LM				ACRO				SM							
				CHR	(%)	ISO	(%)	CHR	(%)	ISO	(%)	CHR	(%)	ISO	(%)	(No.)	%	(No.)	%
6	0.00	3	4	4	100.0											4	100.0	0	0.0
	EC 300	11	18	14	77.8	1	5.6	3	16.7							17	94.4	1	5.6
	EC 3000	12	16	12	75.0	2	12.5	1	6.3							13	81.2	3	18.8
	MEC 300	5	7	6	85.7			1	14.3							7	100.0	0	0.0
	MEC 3000	8	9	6	66.7			2	22.2			1	11.1			9	100.0	0	0.0
12	0.00	4	5	4	80.0			1	20.0							5	100.0	0	0.0
	EC 300	8	10	8	80.0			1	10.0			1	10.0			10	100.0	0	0.0
	EC 3000	9	11	7	63.6	1	9.1	3	27.3							10	90.9	1	9.1
	MEC 300	4	4	2	50.0			2	50.0							4	100.0	0	0.0
	MEC 3000	9	10	8	80.0			2	20.0							10	100.0	0	0.0
24	0.00	4	4	2	50.0			1	25.0	1	25.0					3	75.0	1	25.0
	EC 300	14	33	24	72.7	2	6.1	7	21.2							31	93.9	2	6.1
	EC 3000	10	13	8	61.5			4	30.8	1	7.7					12	92.3	1	7.7
	MEC 300	16	17	12	70.6	2	11.8	3	17.6							15	88.2	2	11.8
	MEC 3000	9	11	9	81.8	1	9.1					1	9.1			10	90.9	1	9.1
48	0.00	5	6	6	100.0											6	100.0	0	0.0
	EC 300	11	12	7	58.3	2	16.7			3	25.0					7	58.3	5	41.7
	EC 3000	10	10	8	80.0	1	10.0	1	10.0							9	90.0	1	10.0
	MEC 300 *	49																	
	MEC 3000	12	13	10	76.9	3	23.1									10	76.9	3	23.1
72	0.00	2	2	2	100.0											2	100.0	0	0.0
	EC 300	6	7	4	57.1	1	14.3	2	28.6							6	85.7	1	14.3
	EC 3000	8	13	9	69.2			4	30.8							13	100.0	0	0.0
	MEC 300	7	8	6	75.0			1	12.5			1	12.5			8	100.0	0	0.0
	MEC 3000	7	10	7	70.0			2	20.0	1	10.0					9	90.0	1	10.0

* Number of breaks, types of breaks and chromosomes affected could not be determined due to fragmentation of the chromosomes.

Figure 9. A Normal Diploid Karyotype of the Don Chinese Hamster Fibroblast-like Strain.

NORMAL KARYOTYPE



isochromatid (ISO). Examples of some of the types of breaks are shown in Figures 10, 11, 12 and 13. According to the data presented in the table for the cells with breaks, most of the breaks were of the chromatid type and occurred mostly in the LM chromosomes for all exposure periods. The range of percentages for this combination was 50% - 100%. The ACRO chromosomes were also affected mostly with breaks of the CHR type. Nearly all of the breaks which occurred in the SM chromosomes were of the CHR type, although breakage in these chromosomes was infrequent. The greatest frequency of ISO breaks occurred in the LM chromosomes with the next highest frequency for the ACRO chromosomes. The SM chromosomes were very rarely affected with ISO breaks.

Effects on Chromosome Numbers

The effects of exposure to the two levels of the test chemicals and control treatment on chromosome numerical aberrations are summarized in Table XII. The distribution of the chromosome numbers of the cells into the five groups are shown for each exposure period and treatment. Examples of four of the groupings are shown in Figures 14, 15, 16 and 17. Ethyl carbamate at 300 µg/ml, when compared with control distributions for all five of the exposure periods, showed the most significant effects in altering the chromosome number of the cells. This treatment showed statistically significant results at all exposure periods except the 48-hour exposure period. Ethyl carbamate at 300 µg/ml, reduced the number of approximately "diploid" metaphases but increased the number of "triploid and tetraploid metaphases." At 3000 µg/ml of ethyl carbamate statistically significant differences in the distribution of the metaphases occurred for two of the exposure

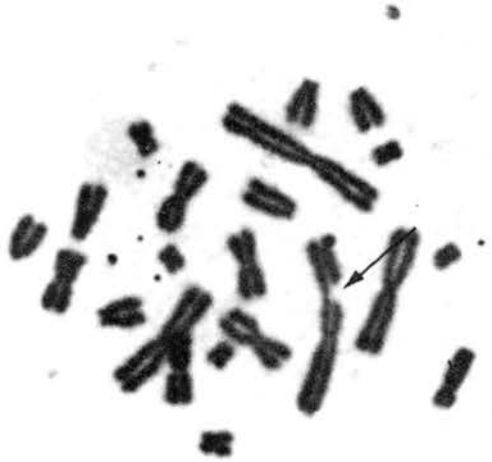
Figure 10. Photomicrograph of a Chinese Hamster Metaphase Chromosome Spread Showing a CHR Break (Arrow) in One of the LM Chromosomes After 24 Hours of Exposure to Ethyl Carbamate (3000 μg/ml).

Figure 11. Photomicrograph of a Chinese Hamster Metaphase Chromosome Spread Showing a CHR Break (Arrow) in One of the ACRO Chromosomes After 6 Hours of Exposure to Methoxyethyl Carbamate (3000 μg/ml).

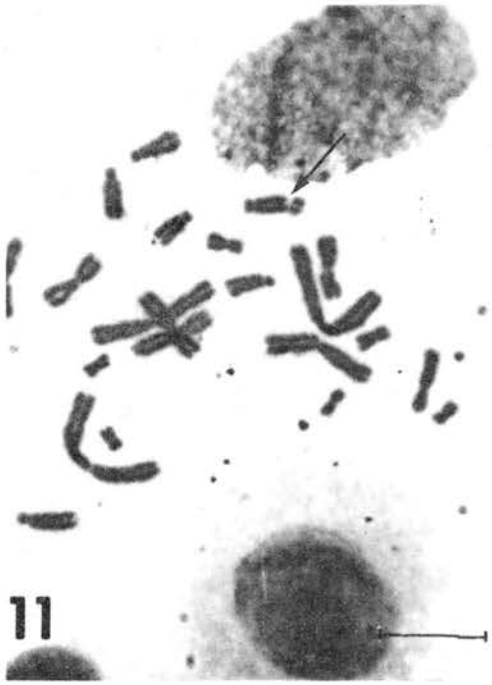
Figure 12. Photomicrograph of a Chinese Hamster Metaphase Chromosome Spread Showing Two Chromosome Fragments (Arrows) produced by an ISO Break at or Near the Centromeric Region of One of the LM Chromosomes After 24 Hours of Exposure to Ethyl Carbamate (300 μg/ml).

Figure 13. Photomicrograph of a Chinese Hamster Metaphase Chromosome Spread Showing Two CHR Breaks in Two Different LM Chromosomes (Arrows) and an Achromatic Gap (G) After 24 Hours of Exposure to Ethyl Carbamate (300 μg/ml).

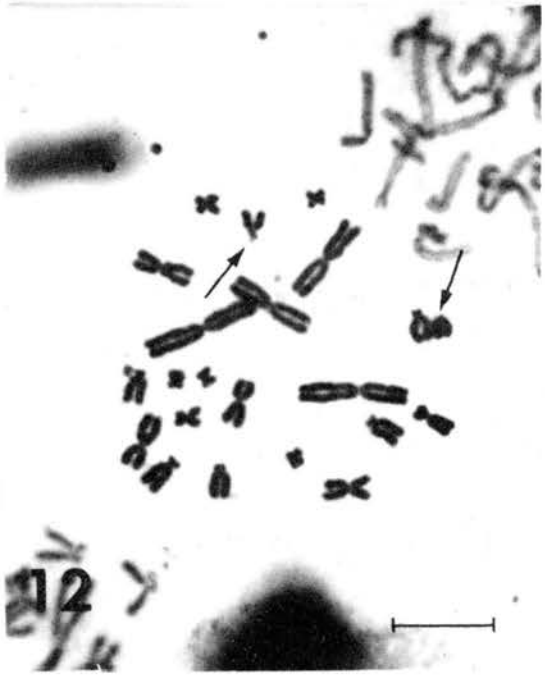
Bars Represent 10 Microns



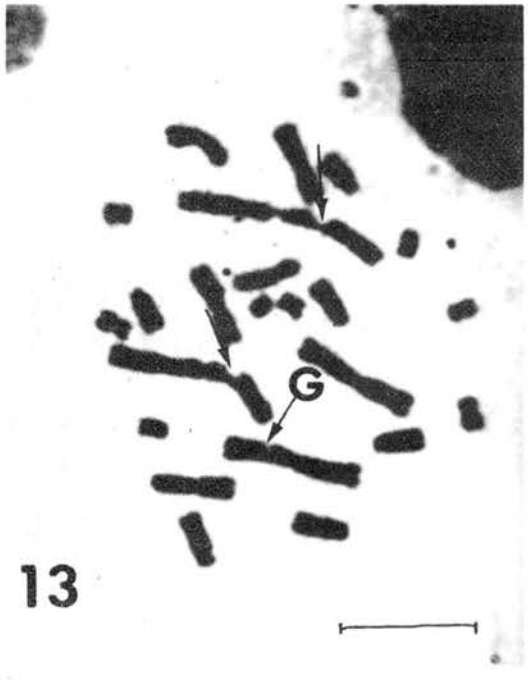
10



11



12



13

TABLE XII

THE EFFECTS OF ETHYL CARBAMATE AND METHOXYETHYL CARBAMATE ON THE DISTRIBUTION OF THE CHROMOSOME NUMBER PER 50 METAPHASES

Length of Exposure (Hours)	Concentrations (µg/ml)	Number of Metaphases					X^2cal	P
		Group I <20	Group II 20-25	Group III 26-29	Group IV 30-50	Group V >50		
6	0.00	0	48	1	1	0		
	EC 300	0	43	0	7	0	37.5208	<0.005
	EC 3000	1	47	0	2	0	2.0208	<0.750
	MEC 300	0	48	0	2	0	2.0000	<0.750
	MEC 3000	0	47	0	3	0	3.0208	<0.750
12	0.00	1	47	0	2	0		
	EC 300	3	34	2	11	0	76.1915	<0.005
	EC 3000	0	43	0	7	0	13.8404	<0.010
	MEC 300	0	45	0	5	0	5.5851	<0.250
	MEC 3000	1	46	0	3	0	0.5213	<0.975
24	0.00	0	48	0	2	0		
	EC 300	0	36	0	14	0	75.0000	<0.005
	EC 3000	1	46	0	3	0	0.5833	<0.975
	MEC 300	2	43	0	5	0	5.0208	<0.500
	MEC 3000	0	45	0	5	0	4.6875	<0.500
48	0.00	0	47	0	3	0		
	EC 300	0	44	0	6	0	3.1915	<0.750
	EC 3000	1	46	0	3	0	0.0213	<0.995
	MEC 300 *							
	MEC 3000	2	45	0	3	0	0.0851	<0.995
72	0.00	1	46	0	2	1		
	EC 300	0	42	0	8	0	20.3478	<0.005
	EC 3000	1	40	0	9	0	26.2826	<0.005
	MEC 300	2	45	0	3	0	2.5217	<0.750
	MEC 3000	0	44	0	6	0	10.0870	<0.050

$X^2_{tab.05(4\ d.f.)}=9.49$

*The distribution of chromosome number in metaphases could not be determined due to fragmented chromosomes.

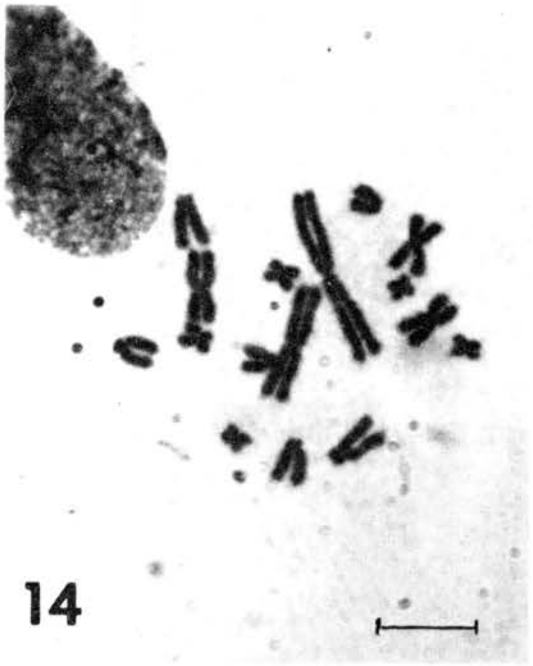
Figure 14. Photomicrograph of a Chinese Hamster Metaphase Chromosome Spread with 16 Chromosomes (Group I).

Figure 15. Photomicrograph of a Chinese Hamster Metaphase Chromosome Spread Showing the Normal Diploid Chromosomal Complements (Group II).

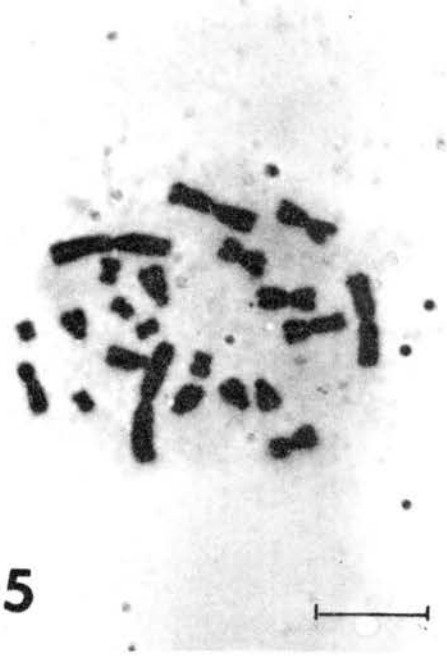
Figure 16. Photomicrograph of a Chinese Hamster Metaphase Chromosome Spread with 27 Chromosomes (Group III), Including an Atypical LM Chromosome (Arrow).

Figure 17. Photomicrograph of a Chinese Hamster Metaphase Chromosome Spread with 43 Chromosomes (Group IV).

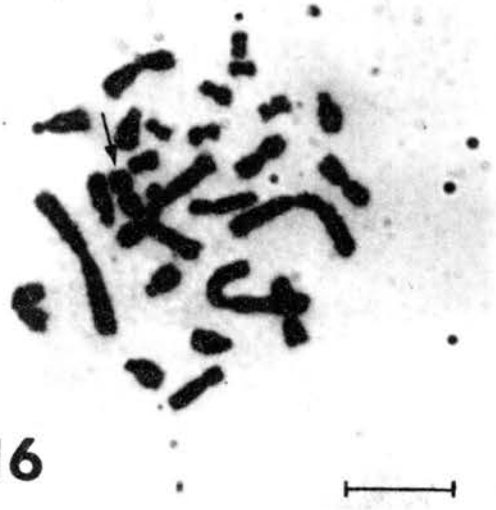
Bars Represent 10 Microns



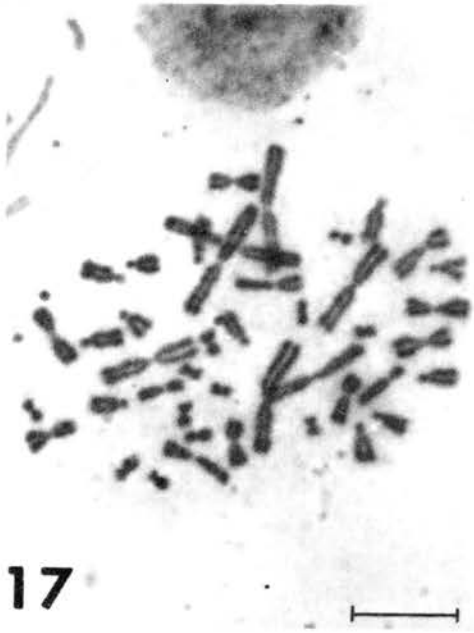
14



15



16



17

periods, 12 and 72 hours. In both periods the distribution of approximately "diploid" metaphases was reduced while the distribution of "triploid and tetraploid metaphases" was increased. Methoxyethyl carbamate at 300 and 3000 μg/ml showed results that differed from the distribution of metaphases for controls, but neither of the treatments gave statistically significant results. However, methoxyethyl carbamate at 3000 μg/ml did show a statistically significant distribution of metaphases when compared with the control at the 72 hour exposure period. The distribution of chromosome numbers for the cells exposed to methoxyethyl carbamate (300 μg/ml) for 48 hours could not be studied due to the fragmentation or pulverization of the chromosomes for this period.

Reversible-Temporary or Irreversible-Permanent Effects of Ethyl Carbamate and Methoxyethyl Carbamate

To determine whether the effects of the two test chemicals were reversible-temporary or irreversible-permanent, one dosage level (300 μg/ml) for each compound was selected for this study. Cells were exposed for 36 hours (treatment period I) to media containing ethyl carbamate or methoxyethyl carbamate. Cells exposed similarly (within period I) were returned to culture in medium without either of the test chemicals for an additional 36-hour period (treatment period II). The criteria selected for the determination of these effects were structural and numerical aberrations and growth rates as determined by metaphase indices within the two treatment periods. Comparisons were also made from the chromosome data between the two treatment periods (I and II) for each treatment. No comparisons of the metaphase

indices were made between the two sample periods. The results presented in this section are given under three main headings for the effects on chromosomal breakage, chromosome number, and metaphase indices.

Effects on Chromosomal Breakage:

The effects of the two experimental and control treatments are summarized in Table XIII. These relationships are expressed in a graphic form for clearer interpretations in Figure 18, where the number of cells with breaks per 10 cells are plotted as a function of each exposure period. Also shown in Figure 18 are the standard errors (S.E.) of each mean. The standard errors are represented by vertical lines in each bar. The table and figure show the number of cells with breaks per 50 cells for each of the duplicate treatments (control and experimentals) for both exposure periods. Within treatment period I, the duplicate cultures treated with medium lacking either of the compounds showed 2/50 and 1/50 cells with breaks respectively. Similarly, for cultures treated with ethyl carbamate (300 μg/ml) 9/50 and 12/50 cells, respectively, showed breaks. At 300 μg/ml methoxyethyl carbamate, for the same period, 11/50 and 8/50 cells demonstrated breaks. Within treatment period II, in the absence of either of the compounds, the numbers of cells with breaks were 4/50 and 3/50, respectively, for the duplicate cultures. For the duplicate cultures that were treated with ethyl carbamate at 300 μg/ml (period I) then returned to normal medium (period II) the numbers of cells with breaks were 10/50 and 8/50, respectively. Likewise, for the cells previously treated with methoxyethyl carbamate, then returned to normal medium, the number of

cells with breaks were 3/50 and 3/50, respectively.

TABLE XIII

EFFECTS OF ETHYL CARBAMATE AND METHOXYETHYL CARBAMATE ON CHROMOSOME BREAKAGE FOR EACH DUPLICATE CULTURE DURING TWO DIFFERENT 36-HOUR TREATMENT PERIODS

Treatments	Concentrations	Cells With Breaks (No./50 Cells)	Cells With Breaks (%)	Cells With Breaks (Mean No./50 Cells)	S.E.
ON COMPOUND MEDIA (36 Hours)	0.00-1	2	4	1.5	0.50
	0.00-2	1	2		
	EC 300-1	11	22	9.5	1.50
	EC 300-2	8	16		
	MEC 300-1	9	18	10.5	1.50
	MEC 300-2	12	24		
ON CONTROL MEDIA (36 Hours)	0.00-3	4	8	3.5	0.50
	0.00-4	3	6		
	EC 300-3	10	20	9.0	1.00
	EC 300-4	8	16		
	MEC 300-3	3	6	3.0	0.00
	MEC 300-4	3	6		

For comparison of the data obtained within treatment period I or II, analysis of variance and F-tests were used (Table XIV). Analysis of variance and F-tests revealed that statistically significant dif-

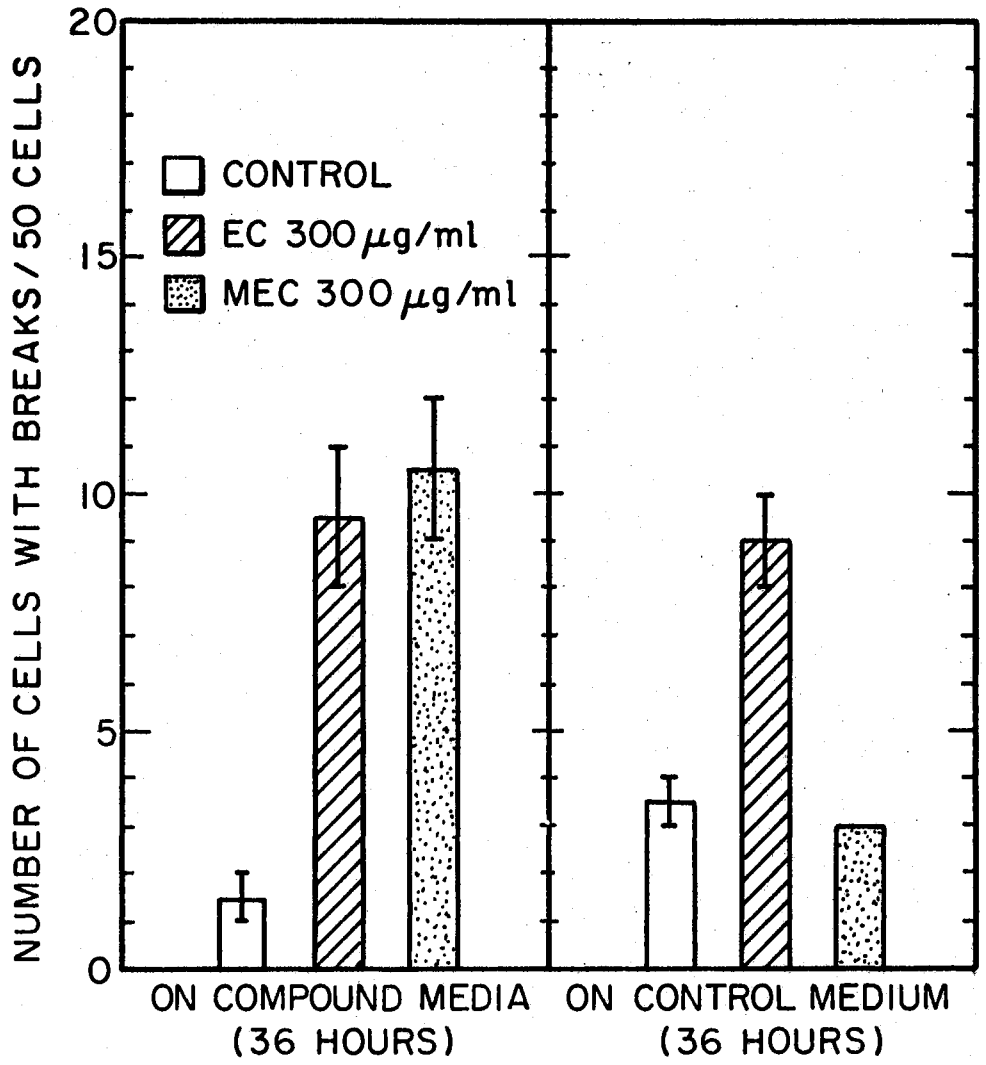


Figure 18. A Comparison of the Effects of Ethyl Carbamate and Methoxyethyl Carbamate on Chromosome Breakage per 50 Cells for Two Different 36-hour Treatment Periods.

ferences existed between the treatments within both of the treatment periods. In further analysis with the t-test on treatment period I, both ethyl carbamate and methoxyethyl carbamate showed statistically significant numbers of cells with breaks when compared with the duplicate controls. However, there existed no significant difference between the treatments with ethyl carbamate and methoxyethyl carbamate for this period. Within treatment period II, the duplicate cultures which had been previously treated with ethyl carbamate at 300 μg/ml showed more cells per 50 with breaks than either the controls or the cultures previously treated with methoxyethyl carbamate at the same level. These differences were statistically significant. No statistical difference was observed between the controls and the cultures previously treated (within period I) with methoxyethyl carbamate. Analysis of variance and F-tests were also used for making comparisons between the effects of the control and experimental treatments between periods I and II (Table XV). For these comparisons the hypotheses tested were $\mu_C I = \mu_C II$, $\mu_E I = \mu_E II$, and $\mu_M I = \mu_M II$. In other words, based on these hypotheses, no significant differences existed between the effects of exposure to a compound for 36 hours and those present after exposure to normal medium for an additional 36 hours. The hypothesis of $\mu_C I = \mu_C II$ and $\mu_E I = \mu_E II$ were accepted, but the hypothesis of $\mu_M I = \mu_M II$ was rejected.

A summary of the distribution of the number of breaks, types of breaks and kinds of chromosomes in which the breaks occurred is presented in Table XVI. Within period I, in the duplicate cultures treated with medium without either of the compounds, most of the breaks were of the chromatid type and they occurred more frequently in the LM chromosomes.

TABLE XIV

ANALYSIS OF VARIANCE FOR THE EFFECTS OF ETHYL CARBAMATE AND METHOXYETHYL CARBAMATE (300 μg/ml) ON THE NUMBER OF CHROMOSOME BREAKS PER FIFTY CELLS WITHIN TWO DIFFERENT TREATMENT PERIODS

Source of Variation	df	SS	MS	F_{cal}
Total	11	165.6667		
Treatments	5	153.6667		
Treatments in Period I	2	97.3333	48.6667	24.3333
Treatments in Period II	2	44.3333	22.1667	11.0833
Periods (Averaged over Treatments)	1	12.0000		
Error	6	12.0000	6.0000	

$F\ tab_{.05(2,6)}=5.14$

Treatment Period I	$\bar{X}_C I = 1.5$	$\bar{X}_E I = 9.5$	$\bar{X}_M I = 10.5$*
Treatment Period II	$\bar{X}_M II = 3.0$	$\bar{X}_C II = 3.5$	$\bar{X}_E II = 9.0$*

*A line under any two means ($\bar{X}$'s) indicates no significant difference between the two treatments.

One of the 4 breaks (25%) observed for the cells which demonstrated breaks was ISO in the LM and one was CHR in the ACRO, respectively. For ethyl carbamate and methoxyethyl carbamate the majority of the cells with breaks had most breaks of the CHR type in the LM chromosomes with 79.3 and 84.0 percent, respectively. Ethyl carbamate caused more ISO breaks in the LM chromosomes than did methoxyethyl carbamate.

Ethyl carbamate and methoxyethyl carbamate caused only CHR breaks in the ACRO chromosomes. Both showed CHR breaks in the SM chromosomes with 3.4% for ethyl carbamate and 4.0% for methoxyethyl carbamate. For treatment period I, 75-90% of all of the cells with breaks showed breaks of the CHR type. Four to twenty-five percent showed ISO breaks.

TABLE XV

ANALYSIS OF VARIANCE FOR THE EFFECTS OF ETHYL CARBAMATE AND METHOXYETHYL CARBAMATE (300 μg/ml) ON THE NUMBER OF CHROMOSOME BREAKS PER FIFTY CELLS BETWEEN TWO DIFFERENT TREATMENT PERIODS

Source of Variation	df	SS	MS	F_{cal}
Total	11	165.6667		
Treatments	5	153.6667		
Periods in Treatment (Control)	1	4.0000	4.0000	2.0000
Periods in Treatment (EC)	1	0.2500	0.2500	0.1250
Periods in Treatment (MEC)	1	56.2500	56.2500	28.1250
Treatments (Averaged over Periods)	2	93.1667		
Error	6	12.0000	2.0000	

$F\ tab_{.05(1,6)} = 5.99$

Ethyl carbamate and methoxyethyl carbamate caused only CHR breaks in the

TABLE XVI

DISTRIBUTION OF THE TYPES OF CHROMOSOME BREAKS AND KINDS OF CHROMOSOMES AFFECTED

Treatment	Metaphases Observed (No.)	Concentrations (μg/ml)	Cells With Breaks (No.)	Breaks (No.)	Chromosomes Affected and Types of Breaks												Total CHR Breaks		Total ISO Breaks	
					LM				ACRO				SM							
					CHR	%	ISO	%	CHR	%	ISO	%	CHR	%	ISO	%	(No.)	%	(No.)	%
ON COMPOUND MEDIA (36 Hours)	100	0.00 - 1 and 2	3	4	2	50.0	1	25.0	1	25.0							3	75.0	1	25.0
	100	EC300 - 1 and 2	19	29	23	79.3	3	10.3	2	6.9			1	3.4			26	89.7	3	10.3
	100	MEC300 - 1 and 2	21	25	21	84.0	1	4.0	2	8.0			1	4.0			24	96.0	1	4.0
ON CONTROL MEDIUM (36 Hours)	100	0.00 - 3 and 4	7	7	1	44.3	1	14.3	5	71.4							6	85.7	1	14.3
	100	EC300 - 3 and 4	18	24	15	62.5	5	20.8	3	12.5	1	4.2					18	75.0	6	25.0
	100	MEC300 - 3 and 4	6	13	10	76.9			3	23.1							13	100.0	0	0.0

ACRO chromosomes. Both showed CHR breaks in the SM chromosomes with 3.4% for ethyl carbamate and 4.0% for methoxyethyl carbamate. For treatment period I, 75-96% of all of the cells with breaks showed breaks of the CHR type. Four to twenty-five percent showed ISO breaks.

Within period II, the duplicate cultures of cells treated with medium lacking either of the compounds showed CHR breaks in both the LM and ACRO chromosomes. Most of the CHR breaks occurred in the ACRO chromosomes (71.4%). Only one of the cells, showed an ISO break. The ISO break in this case occurred in one of the LM chromosomes. No breaks were in the SM chromosomes. For ethyl carbamate and methoxyethyl carbamate, the majority of the cells with breaks show CHR-type breaks which occurred more frequently in the LM chromosomes. Ethyl carbamate caused ISO breaks in the LM chromosomes, but the same was not observed for methoxyethyl carbamate. Ethyl carbamate and methoxyethyl carbamate also caused breaks in the ACRO chromosomes. For treatment period II, 75-100% of all of the cells which showed breaks had CHR-type breaks. The percentage range of ISO breaks for all treatments was 0.0%-25.0%.

Effects on Chromosome Number:

The effects of the exposure for both treatment periods (I and II) on the chromosome number of Chinese hamster fibroblasts are shown in Table XVII. These results are given in a graphic form in Figure 19 where the number of metaphases per 100 are plotted against the groupings (I, II, III, IV and V) for each treatment period. Within treatment period I, cells treated with ethyl carbamate showed a difference in the distribution of the chromosome number when compared with the control treatment. The chi-square value obtained from this comparison was

TABLE XVII

EFFECTS OF ETHYL CARBAMATE AND METHOXYETHYL CARBAMATE ON THE DISTRIBUTION OF THE CHROMOSOME NUMBER FOR TWO 36-HOUR TREATMENT PERIODS

Treatments	Concentrations (µg/ml)	Number of Metaphases					X^2_{cal}	P
		Group I 20 (No.)	Group II 20-25 (No.)	Group III 26-29 (No.)	Group IV 30-50 (No.)	Group V 50 (No.)		
On Compound Media (36 Hours)	0.00-1 and 2	1	88	1	9	1		
	EC300-1 and 2	0	72	4	21	3	21.3534	<0.005
	MEC300-1 and 2	1	88	0	10	1	1.1111	<0.900
On Control Media (36 Hours)	0.00-3 and 4	1	94	0	5	0		
	EC300-3 and 4	0	90	0	10	0	6.1702	<0.250
	MEC300-3 and 4	1	94	0	5	0	0.0000	<0.995

$X^2_{tab.05(4\ d.f.)} = 9.49$

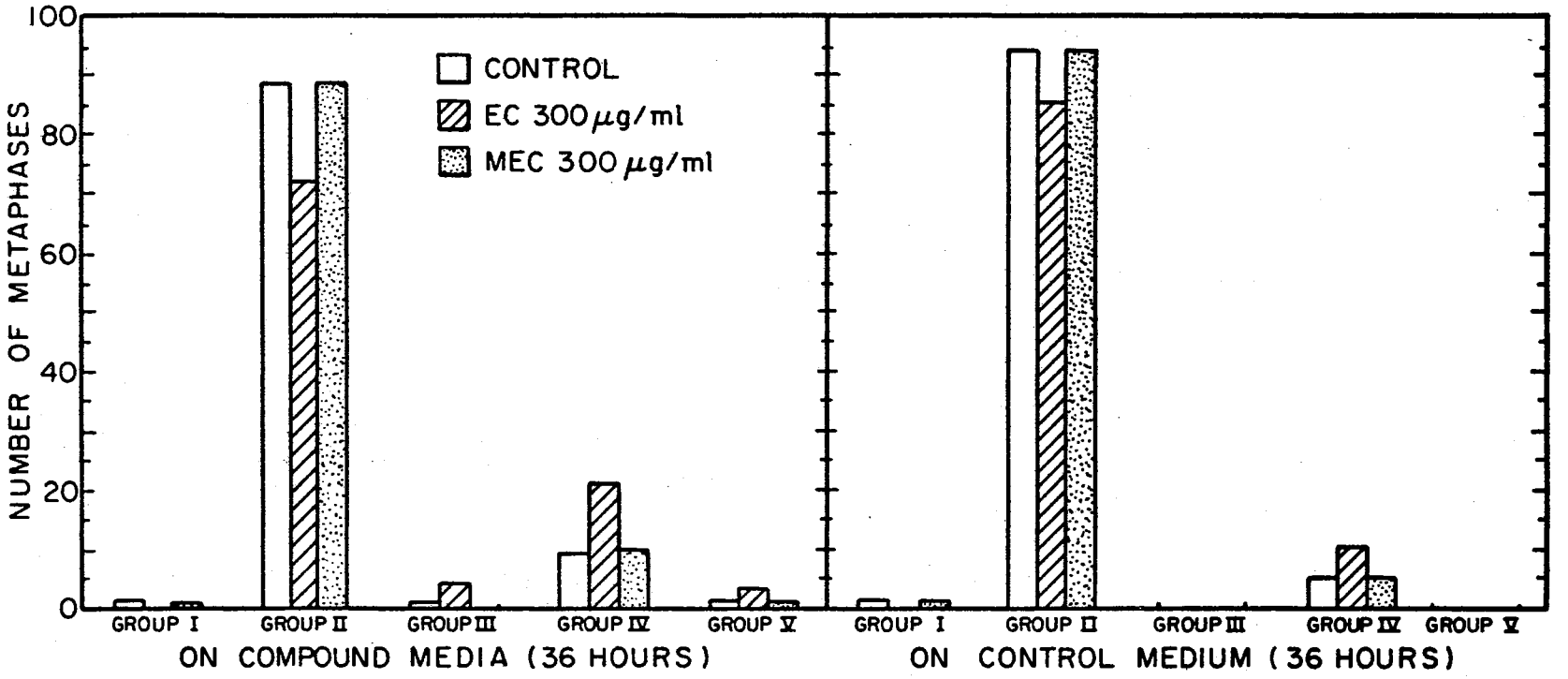


Figure 19. A Comparison of the Effects of Ethyl Carbamate and Methoxyethyl Carbamate on the Distribution of the Chromosome Number per 100 Metaphases for Two Different 36-hour Treatment Periods.

highly significant. Treatment with ethyl carbamate reduced the frequency of metaphases with the approximately "diploid" karyotype and increased the frequency of metaphases of the "triploid and tetraploid" category. Methoxyethyl carbamate, within treatment period I, showed no statistically significant difference in the distribution of metaphases when compared with the control distribution. Within treatment period II neither of the experimental distributions (ethyl carbamate or methoxyethyl carbamate) of metaphases differed statistically from the control distribution.

The effects of the treatments on the growth rate of Chinese hamster fibroblasts, as attested by metaphase index data for both periods, are summarized in Table XVIII. For clarity these relationships are presented graphically in Figure 20 where the number of cells in colcemid metaphase per thousand are plotted against the treatment periods for each treatment. Within period I, the number of cells per 1000 in colcemid metaphase for the duplicate controls was 39 and 59, respectively, with a mean of 49.0. The number of cells per 1000 in colcemid metaphase after a 36-hour exposure to ethyl carbamate were 98 and 70, respectively, for the duplicate cultures. The mean ratio of these duplicates was 84.0/1000. For the methoxyethyl carbamate duplicate cultures, the number of cells per 1000 in colcemid metaphase was 54 and 53, respectively, with a mean of 53.5 Within period II, the duplicate control cultures showed 22 and 16 cells per 1000 in colcemid metaphase and a mean of 19.0. For the duplicate cultures that were treated with ethyl carbamate (period I) and normal medium (period II) the number of cells per 1000 in colcemid metaphase was 29 and 25 with a mean of 27. For the duplicate cultures that were treated with methoxyethyl carbamate

(period I) and normal medium (period II), the number of cells in colcemid metaphase per 1000 was 23 and 22 for a mean of 22.5. For treatment period II, the number of cells in colcemid metaphase was reduced for all treatments when compared with the results obtained for treatment period I.

TABLE XVIII

METAPHASE INDICES FOR THE DUPLICATE CULTURES OF THE SAME TREATMENT FOR TWO DIFFERENT TREATMENT PERIODS

Treatments	Concentrations (µg/ml)	Metaphases Observed (No./1000)	(%)	Mean No./1000	S.E.
On Compound Media (36 Hours)	0.00-1	39	3.9	49.0	10.00
	0.00-2	59	5.9		
	EC 300-1	98	9.8	84.0	14.00
	EC 300-2	70	7.0		
	MEC 300-1	54	5.4	53.5	0.50
	MEC 300-2	53	5.3		
On Control Medium (36 Hours)	0.00-3	22	2.2	19.0	3.00
	0.00-4	16	1.6		
	EC 300-3	29	2.9	27.0	2.00
	EC 300-4	25	2.5		
	MEC 300-3	23	2.3	22.5	0.50
	MEC 300-4	22	2.2		

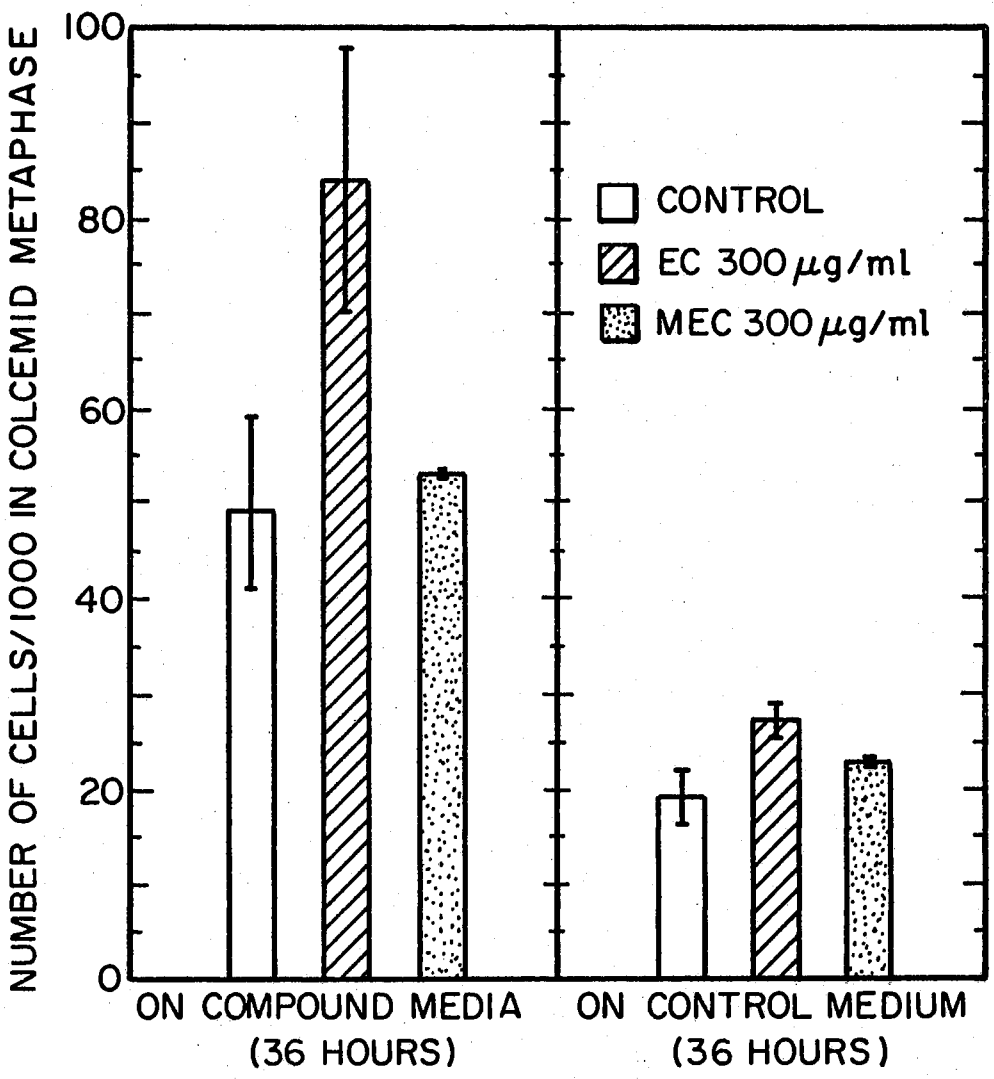


Figure 20. A Comparison of the Effects of Ethyl Carbamate and Methoxyethyl Carbamate on the Number of Cells/1000 in Colcemid Metaphases for Two Different 36-hour Treatment Periods.

For the evaluation and comparison of the data obtained within treatment period I and II, analysis of variance and F-tests were used (Table XIX). Analysis of variance and F-tests showed that statistically significant differences existed between the treatments within period I. The same procedures revealed that within treatment period II no statistically significant differences existed between the treatments. From further analysis with the use of a t-test, ethyl carbamate showed differences which were statistically significant when compared with the effects of methoxyethyl carbamate or control treatment for treatment period I. The effects of methoxyethyl carbamate, within treatment period I, did not differ statistically from the control treatment. Within treatment period II, none of the effects for any of the treatments was statistically significant. However, the number of cells in colcemid metaphase per 1000 which received treatment with ethyl carbamate (period I) was higher than either methoxyethyl carbamate or control treatment.

TABLE XIX

ANALYSIS OF VARIANCE FOR THE EFFECTS OF ETHYL CARBAMATE AND METHOXYETHYL CARBAMATE (300 μg/ml) ON THE METAPHASE INDICES WITHIN TWO DIFFERENT TREATMENT PERIODS

Source of Variation	df	SS	MS	F_{cal}
Total	11	6775.0000		
Treatment Combinations	5	6156.0000		
Treatments in Period I	2	1450.3333	725.7666	7.029
Treatments in Period II	2	64.3333	32.1666	0.3118
Periods (Averaged over Treatments)	1	4641.3333		
Error	6	619.0000	103.1667	

$F\ tab_{.05(2,6)} = 5.14$

Treatment Period I	$\overline{X}_C I = 49.0$	$\overline{X}_M I = 53.5$	$\overline{X}_E I = 84.0$	*
Treatment Period II	$\overline{X}_C II = 19.0$	$\overline{X}_M II = 22.2$	$\overline{X}_E II = 27.0$	*

* A line under any two means indicates no significant difference between the two treatments.

CHAPTER IV

DISCUSSION

Growth Rate Experiments

A comparison of the effects of ethyl carbamate and methoxyethyl carbamate on the growth rate of Chinese hamster fibroblasts, with data obtained from Coulter counter enumerations, revealed that the effects of the two compounds could be grouped. The grouping of the effects results in three categories; 1) stimulation of growth rates, 2) retardation of growth rates, and 3) lethality. Expression of these effects were dependent upon the dosage levels of the two compounds to which the cells were exposed. The same type of relationship of the effects of various dosage levels of ethyl carbamate and response phenomena as indicated in the present study have also been reported in the literature. Bucher (1949 b) concluded that the action of ethyl carbamate depends on the dose, length of action, and biological cellular resistance. Johnson (1942) and Burger (1968) observed that growth rate and other characteristics in certain bacteria were dependent upon the dosage level of ethyl carbamate tested. Both of these researchers found that low concentrations of the compound stimulated growth and high concentrations tended to inhibit the growth of bacteria. Similar observations were reported by Morgans (1968) using fish cell lines.

Based on the means of the growth ratios for experiments A, B, C

and D in which various concentrations of ethyl carbamate were tested, the dosage levels less than 3000 µg/ml caused stimulation of growth. Similarly for experiments E, F and G in which methoxyethyl carbamate was used, accelerated growth rates were obtained for cells exposed to each of the dosage levels inclusive of 3000 µg/ml, but exclusive of 30,000 µg/ml. The effects of the two compounds at 3000 µg/ml differed sharply. Cells exposed to ethyl carbamate at this concentration showed a retarded or decreased growth rate. This reduced growth rate, however, was not statistically significant when compared with the growth rate of control cells. Cells exposed to methoxyethyl carbamate at the same concentration showed a growth rate greater than two times that of their respective control. For both sets of experiments, similar trends in the effects of the two compounds were observed for concentrations of 30 and 300 µg/ml. In each experiment set a more stimulated growth rate (higher growth ratio) occurred at 30 µg/ml than was found at 300 µg/ml, but in neither case was the difference statistically significant. Lethal effects for both compounds were observed at 30,000 µg/ml in the two sets of experiments. In the two sets of experiments, very little differences could be discerned by comparing the effects of same dosage levels of the compounds with reference to the secondary characteristics of pH (color change in the media as indicated by the phenol red indicator), cell attachment, degree of confluency and cellular morphology.

Further comparisons were made between the effects of the two compounds on the growth rates of the cells in separate, but similar, experiments. In each of these experiments, the effects of concentrations at 6000, 9000, 12,000 and 15,000 µg/ml were tested. Experiment H was

designed for ethyl carbamate and Experiment I for methoxyethyl carbamate. Similarities and dissimilarities of the effects of the above dosage levels for both compounds were observed in the two experiments. Both compounds at 6000 and 9000 µg/ml caused similar effects of retardation of the growth rates. As the dosage level increased, the rates of growth of the cells decreased. For the higher concentrations tested (12,000 and 15,000 µg/ml) cells exposed to methoxyethyl carbamate continued to show reduced growth rates. Ethyl carbamate at dosage levels of 12,000 and 15,000 µg/ml showed a direct proportional relationship between the concentration tested and percentage of lethality. However, all concentrations of methoxyethyl carbamate tested in Experiment I were considered sublethal.

By comparing the results obtained in the different sets of experiments (above) with reference to the dosage levels of the two compounds tested, it is evident that the lower concentrations tended to stimulate or promote the growth rates while higher dosages either retarded growth rates or were partially or wholly lethal. The data from these experiments also indicate that methoxyethyl carbamate in the lower concentrations tested (30 to 3000 µg/ml) had a more stimulative effect than ethyl carbamate in the corresponding dosage levels. Methoxyethyl carbamate in the higher concentrations tested (6000 to 15,000 µg/ml) caused a greater reduction in the growth rates than did ethyl carbamate in similar dosages.

Numerous investigations, by other researchers, have been conducted on the effects of ethyl carbamate on mitotic activity and growth rates of various biological materials (<u>in vivo</u> and <u>in vitro</u>). As with the present research, the reports of the effects of ethyl carbamate on the

phenomenon of growth of these biological materials can be grouped or categorized. Decreases and increases of the growth rates after exposure to the compound have been reported. Lethal effects brought on by exposure to the compound have also been reported.

The results of the present growth rate studies tend, for the most part, to confirm results obtained by other investigators using different biological systems. Bastrop-Madsen (1949) found that concentrations of ethyl carbamate ranging from 0.66% (6600 µg/ml) to 1.2% (12,000 µg/ml) caused chick fibroblast cells to decrease in mitotic activity. The findings of the present research tend to support the data of the latter researcher for both compounds in similar concentration levels (6000 - 12,000 µg/ml). However, in the present study ethyl carbamate demonstrated some lethal effects on Chinese hamster fibroblasts at 12,000 µg/ml. Geiersbach (1939) concluded that inhibition of mitosis occurred when chick fibroblast cultures were treated with the compound in the 0.50% - 0.75% range. Patterson and Thompson (1949) demonstrated with chick fibroblasts that when ethyl carbamate was used with X-rays, the impact of the X-rays was diminished if the compound was added to the culture medium in a concentration of 0.1%, which in itself would reduce mitosis. The results obtained in the present research by the action of ethyl carbamate and methoxyethyl carbamate at a concentration of 9000 µg/ml (the closest dosage to 0.1%) also retarded the rates. Morgans (1968) working with two cultured fish cell lines found that ethyl carbamate at 0.6% and 0.9% caused the rates of cell division to decrease. Lasnitski (1949) tested the effects of ehtyl carbamate at 0.4% (4000 µg/ml) on a number of normal mouse cell cultures and found that they showed a reduction of growth and a fall

in the number of cell divisions to approximately half of the control value. In the present study, the closest dosages of ethyl carbamate tested to the one tested by Lasnitzki were 3000 and 6000 µg/ml, which reduced the growth rate of Chinese hamster cells about 10% and 30%, respectively. Methoxyethyl carbamate at 6000 µg/ml caused about a 59% reduction compared with its control rate. Further studies in which ethyl carbamate has caused a lowered, reduced mitotic, or growth rate were made with cats (Moeschlin and Bodmer, 1950); patients who suffered from chronic myeloid leukemia (Dustin, 1949); corneal cells of rats (Guyer and Clause, 1947 a and b); marrow myeloid cells in mice (Kirchbaum and Lu, 1947); root tips of Vicia faba and Allium cepa (Hohl, 1947); and intestinal cells of mice (Dustin, 1947). Battle and Hisaoka (1952) reported retardation of growth and differentiation of embryonic structures of the Zebra fish with ethyl carbamate in concentrations ranging from 0.25% to 1.25%.

In addition to causing a decreased growth rate on various biological materials, ethyl carbamate has been shown to have stimulatory effects. Lasnitzki (1949) reported that in mouse tumor cell cultures of C 57 sarcoma and adenocarcinoma 63, a stimulation of growth and mitosis to 2 to 4 times that of the untreated controls was observed.

To parallel these observations with the findings of the present study for both compounds, since each stimulated growth rates in the lower concentrations tested, might suggest, based on growth rate characteristics alone, that the treated cell cultures may have been transformed into "tumor-like cultures." However, Harbel (1968) has pointed out that the most conclusive evidence for such an assumption of transformation would be the production of tumors on transplantation

of cells to immunologically compatible hosts. This aspect of the alterations of cell populations, however, was not pursued in the present study. Other evidences of increased growth or mitotic rates after exposure to ethyl carbamate were found by Rosin (1951) in the bone marrow of normal mice, and Morgans (1968) in fish fibroblastic and epithelial cell lines.

Lethal effects of ethyl carbamate on various biological systems have also been reported. Toplin (1959) tested the effects of the compound on human cell cultures (HeLa and others) and concluded that the lethal end point for the compound was greater than a dosage of 10,000 μg/ml. Globerson and Auerbach (1965) found that ethyl carbamate at 10,000 μg/ml was lethal to lymphocytes, alveolar tissue and connective tissue explants after 4 days of exposure.

Doubling Time Experiments

The comparisons of the effects of ethyl carbamate and methoxyethyl carbamate on the doubling time of Chinese hamster fibroblast populations were obtained from two separate, but similar, experiments. In the experiment in which 300 μg/ml of each compound were compared, slight differences were noted in the amounts of time required for the treated populations to double for the individual doubling periods. However, when the means of the individual doubling times for each compound were compared, no statistical significance was found. When the mean doubling time for each of these populations was compared with that of the control populations, again, no statistical significance was observed. Based on the data from this experiment neither compound at 300 μg/ml significantly affected the mean doubling time of the cell

populations. In this experiment, however, it was noted that for some unexplainable reason, both ethyl carbamate and methoxyethyl carbamate caused a decrease in the amount of time for the cell populations to double in the first doubling period compared with the control. A possible explanation for the difference between doubling times of the treated and control populations within the first period, could be that the presence of the compounds in the media influenced the rate of uptake of the nutritive materials. However a valid explanation of these phenomena was not pursued in the present research.

The data from this experiment as shown in Figure 3, tend to support the results previously mentioned in the 72-hour growth rate studies. This observation is based wholly on the assumption that if the trends of the growth curves (Figure 3) shown in 48 hours for all populations were extrapolated to 72 hours, the number of cells at this point for both of the treated populations would exceed that of the control. This extrapolated increase in the number of cells for both of the treated populations over the extrapolated number of cells for the control, would indicate stimulated growth rates. The validity of this assumption would hold only if the number of cells at time 0 (baseline) and 72 hours were taken into account as was the case in the growth rate studies. Further, if this extrapolation were made, it would be evident that the growth rate of methoxyethyl carbamate-treated cells would exceed those of both ethyl carbamate-treated and control cells.

In the experiment in which the effects of 3000 μg/ml of each of the compounds were tested and compared, slight differences were noted in the times of doubling for the treated populations within the various periods. However, for valid comparison of the data from this experi-

ment only two doubling times were chosen for treated and untreated populations. When the mean doubling time of the control populations was compared with the mean doubling time for each of the treated populations, no statistical difference was observed. The overall trends of the growth curves (Figure 4) in this experiment, like the one mentioned above, tend to show the same sort of results as the growth rate studies. The growth curve from populations of cells treated with methoxyethyl carbamate at 3000 µg/ml showed an increase in the number of cells over the control (stimulated growth) and an increase in the number of times doubling occurred. For populations of cells treated with ethyl carbamate at the same dosage level a decrease in the number cells and the number of doubling times were observed.

The Effects of Ethyl Carbamate and Methoxyethyl Carbamate on the Chromosomes of Chinese Hamster Fibroblasts

The results of this researach clearly indicate that both ethyl carbamate and methoxyethyl carbamate (at 300 and 3000 µg/ml) induce chromosomal breaks in Chinese hamster fibroblasts after various periods of exposure. Cultures treated with ethyl carbamate at 300 µg/ml had statistically significant numbers of cells with chromosome breaks as early as 6 hours after initial exposure. This would tend to suggest that the action of this compound on chromosomal breakage is relatively non-delayed. Further, the compound at this dosage level continued to cause chromosomal breaks in statistically significant numbers of cells for all of the other periods of exposure (12, 24, 48 and 72 hours). After reaching a maximum peak for the number of cells with chromosome breaks at 24 hours, the effects of the compound tended to decrease

with time (Figure 5). By contrast to the effects of ethyl carbamate at 300 µg/ml, methoxyethyl carbamate at the same concentration demonstrated a delayed action for about 12 or more hours before it produced significant numbers of cells with chromosomal breaks. Statistically significant numbers of cells with chromosome breaks as affected by the compound at 300 µg/ml was first observed after 24 hours of exposure, then reached a maximum at 48 hours before declining after 72 hours of exposure. Methoxyethyl carbamate at 300 µg/ml continued to produce statistically significant numbers of cells with chromosome breaks for the latter two exposure periods after its initial delay. After 48 hours of exposure, methoxyethyl carbamate at 300 µg/ml caused multiple chromosomal breaks and highly fragmented or pulverized chromosomes. These effects were also observed after 72 hours of exposure to the compound, but the frequency of their occurrence was drastically diminished.

The delayed effects of methoxyethyl carbamate (300 µg/ml) on chromosome breakage might have at least two possible explanations although the mechanisms for these effects were not pursued in the present study. One possibility for the delay, at this dosage level, is that the compound itself could be relatively inactive on chromosome breakage, but might act through the production of an intermediate breakdown product which in itself may be more effective, but may require several hours before it is produced in sufficient quantity to cause structural chromosome damage. Another possible explanation for the delay, in the production of chromosomal breaks, lies in the fact that if the compound does not act through a breakdown metabolite, its effects may be cumulative. This would mean that the cells could poss-

accumulate large quantities of the compound before the deleterious effects of chromosomes appear.

Ethyl carbamate at 3000 µg/ml, as at 300 µg/ml, caused chromosomal breakage in statistically significant numbers of cells with chromosome breaks as early as 6 hours after initial exposure. The action of the compound at this level was also non-delayed. Ethyl carbamate at 3000 µg/ml resulted a rather consistent pattern of statistically significant numbers of cells with breaks for the remaining periods of exposure. Methoxyethyl carbamate at this dosage level also caused statistically significant numbers of cells to show chromosome breaks after as little as 6 hours of exposure. Thus its action was also non-delayed. The compound continued to produce significant numbers of cells with chromosome breaks for the remaining periods. However, after 48 hours of exposure, the compound showed its maximum effectiveness with reference to causing chromosomal breaks.

By comparison, the effects of methoxyethyl carbamate at 300 µg/ml on chromosome breakage were considered delayed, but its effects at 3000 µg/ml were non-delayed. This observation would tend, then, to add some supportive evidence to the second hypothesis advanced above with reference to the delayed effects produced by methoxyethyl carbamate at 300 µg/ml. Methoxyethyl carbamate at 3000 µg/ml, i.e., a ten-fold increase in the level of concentration over methoxyethyl carbamate at 300 µg/ml, may have been present in sufficient strength initially to produce chromosomal damage without its accumulation; or accumulation may occur more rapidly in higher concentrations in the medium.

Both compounds at the two levels tested (300 and 3000 µg/ml)

showed specificity for the types of breaks and the kinds of chromosomes affected. For both compounds at each dosage level, most of the breaks were of the CHR type and occurred more frequently in the LM and ACRO chromosomes. SM chromosomes were very rarely broken by either compound at either concentration tested. ISO breaks were caused by both of the compounds and occurred more frequently in the LM chromosomes than in the ACRO chromosomes. No breaks of this type were found in the SM chromosomes. The distribution of CHR and ISO breaks seems to be related to the length of the chromosomes. The longer the chromosomes the greater the frequency of chromosome breaks for both of the compounds and both dosage levels.

The results obtained from the present study with reference to the effects of ethyl carbamate in causing chromosomal structural damage tend to support and add to the findings of other investigators. Many reports, by other researchers, have indicated that ethyl carbamate does affect the structural integrity of the chromosomes from a variety of biological materials (plants and animals). As mentioned previously, ethyl carbamate was the first chemical shown to produce structural chromosomal changes (Oehlkers, 1943). Other reports have also indicated that ethyl carbamate causes structural damage to chromosomes in the bone marrow of mice (Rosin, 1951); Walker rat carcinoma (Green and Lushbaugh, 1949) (Boyland and Koller, 1954); the grasshopper Peocilocera picta (Rao, 1961); Vicia faba (Deufel, 1951, 1952) and (Koller, 1954); Oenothera (Oehlkers and Linnert, 1949); four strains of mice (Colnaghi, 1969); primary cultures of mouse embryo lung cells and cell lines of Chinese hamster (Pogosyants, et. al., 1968).

The results of the present research indicate that ethyl carbamate

at 300 µg/ml was effective in the production of numerical chromosomal aberrations. To some extent both ethyl and methoxyethyl carbamates at 3000 µg/ml showed partial effects on numerical aberrations with the former compound being more effective than the latter. Methoxyethyl carbamate at 300 µg/ml did not cause statistically significant alterations in the chromosome number of the exposed cells.

The effects of ethyl carbamate, with reference to numerical aberrations, were considered non-delayed since it produced significant alterations in the chromosome number of the cultured cells as early as 6 hours after initial exposure. The compound at 300 µg/ml caused significant alterations in the distribution of the chromosome number for all of the exposure periods except one (48 hours). The greatest alteration in the distribution of the chromosome number of the observed metaphases occurred by a reduction of the frequency of approximately "diploid" metaphases (Group II) and an increase in the frequency of the "triploid and tetraploid" metaphases (Group IV). Ethyl carbamate at 3000 µg/ml showed similar effects, but these were infrequently or less consistently observed (12 and 72 hours after initial exposure).

Although the causes or mechanisms involved in the production of chromosome numerical chromosome aberrations were not pursued in this investigation, findings of other researchers, using ethyl carbamate, yield some possible clues as to the results obtained. Bastrop-Madsen (1949) using chick fibroblast cultures suggested that the action of ethyl carbamate was to reduce mitotic activity and to inhibit mitosis at metaphase. He did not, however, pursue the effects of the compound on numerical aberrations, but reported the occurrence of three types of abnormal metaphase. Some of the chromosomes failed to become

attached to the equatorial plate, while in some cells the plate was reduced to a chromatin bunch, and further reduction in the size of the plate and disappearance of the spindle occurred. Deufel (1951) using Vicia faba pointed out that even brief treatment with ethyl carbamate produced derangement of the spindle and that movement of the chromosomes was disturbed by damage to the centromere. Based on these results, it appears that such abnormal events occurring in the process of cell division would tend to produce alterations in ploidy. Further such events would also tend to interfere with cell division, but not replication of the chromosomes, and therefore would increase the frequency of metaphases diverging from a normal diploid complement.

Reversible-Temporary or Irreversible-Permanent Effects of Ethyl Carbamate and Methoxyethyl Carbamate

The results of the experiment in which ethyl carbamate and methoxyethyl carbamate at 300 μg/ml were tested and compared with reference to effects being either reversible-temporary or irreversible-permanent revealed numerous differences between the two compounds. In this experiment three parameters of the effects of the compounds were measured; chromosome breakage, changes in chromosome number, and growth rate as determined by the metaphase index method.

With reference to chromosome breakage, both ethyl carbamate and methoxyethyl carbamate (at 300 μg/ml) caused the production of statistically significant numbers of cells with breaks after the initial 36-hour exposure (treatment period I). However, there was no statistical difference between the effects of the two compounds at this time. Cells which received the same treatment (within period I) were returned

to culture in normal medium, lacking either of the compounds, for an additional 36 hours (treatment period II). Analysis of the data from treatment period II, revealed that significant numbers of cells treated with ethyl carbamate (within treatment period I) continued to show chromosomal breaks. The same effects (within treatment period II) with methoxyethyl carbamate treated (period I) cells were not found. With reference to chromosomal breakage, the effects of ethyl carbamate were considered permanent-irreversible, but the effects of methoxyethyl carbamate were considered temporary-reversible. In other words, the effects of ethyl carbamate on the phenomenon of chromosomal breakage, were found independent of the compound's presence after an initial exposure. The same relationship was not true for methoxyethyl carbamate where the effects were dependent upon its presence.

These results, however do not preclude the possibility that the permanency of the effects of ethyl carbamate on chromosome breakage might be ephemeral, but they only suggest that 36 hours after the cessation of treatment with the compound, the effects were still evident. Colnaghi (1969), using four strains of mice demonstrated that thymic tissue during exposure to ethyl carbamate showed chromosomal breaks, acentric fragments, and metacentric ring and unusually short or long chromosomes. He further reported that most of these chromosomal alterations became rare by one week after the termination of ethyl carbamate administration and were absent 5 and 11 weeks later.

The effects of the two compounds on chromosomal numerical aberrations were also studied and compared in this experiment. After the initial 36-hour exposure (treatment period I) only ethyl carbamate caused any significant divergence in the distribution of the chromosome

number for the metaphases. As before (previous chromosome study), ethyl carbamate caused the distribution of the chromosome number of the metaphases to be significantly distorted when compared with the control distribution of metaphases for treatment period I. The compound caused a reduction in the number of the approximately "diploid" metaphases (Group II) and an increase in the number of "triploid and tetraploid" metaphases (Group IV). The effects of methoxyethyl carbamate on the chromosome number within treatment period I did not differ statistically from the controls. These results also substantiate the findings mentioned in the previous chromosome study. Within treatment period II neither of the experimental distributions of metaphases (ethyl carbamate or methoxyethyl carbamate) differed statistically from the control distribution. However, cells treated with ethyl carbamate (treatment period I) still showed a somewhat distorted chromosome number distribution. From these data the effects of ethyl carbamate on altering the chromosome number of Chinese hamster fibroblasts were considered temporary-reversible. Methoxyethyl carbamate was considered ineffective in the production of chromosomal numerical aberrations.

A comparison of the effects of ethyl carbamate and methoxethyl carbamate on the growth rate of Chinese hamster fibroblasts, with data obtained by the metaphase index method, showed that only ethyl carbamate caused a significant increase in the growth rate after the initial 36 hours of exposure (treatment period I). The number of cells in colcemid metaphase was higher for cells treated with ethyl carbamate than was observed for those treated either with methoxyethyl carbamate or medium lacking either compound (control). Within this period, the

effects of methoxyethyl carbamate on the growth of these cells, did not differ statistically from the control. Cells which received the same treatment (within period I) were returned to culture in normal medium, without either of the compounds, for an additional 36 hours (treatment period II). Analysis of the data from treatment period II, revealed that the growth rate of neither of the populations previously treated with ethyl carbamate or methoxyethyl carbamate (period I) differed statistically from that of the control. However, the number of cells in colcemid metaphase, again, was higher for the cells which received prior treatment with ethyl carbamate (period I) than was found for the cells which received either methoxyethyl carbamate or control medium. Based on these data, the effect of ethyl carbamate on the growth rate of these cells was considered ineffective with reference to the growth rate of the cells as attested by the metaphase index procedure.

The reversible effects of ethyl carbamate on the growth rate as demonstrated in this experiment tend to support the findings of Bastrup-Madsen (1949) using chick fibroblasts. He concluded that ethyl carbamate appears not to cause irreversible damage to non-dividing cells. Further he pointed out that after 24 hours of exposure to concentrations capable of inhibiting mitosis, the fibroblasts were able to continue their normal growth when the compound was removed from the cultures. In the present research, the compound did not cause irreversible damage to actively dividing cells.

CHAPTER V

SUMMARY AND CONCLUSIONS

The *in vitro* effects of ethyl carbamate and methoxyethyl carbamate on the growth and chromosomes of Chinese hamster fibroblasts were studied and compared. Specifically, the effects of the two compounds on the growth rate, doubling time, structural and numerical aberrations in chromosomes, and reversible-temporary or irreversible-permanent changes in cell populations were considered.

For experimental work, the Don strain of the Chinese hamster lung was used. Cells of the strain were cultured as monolayers in McCoy's 5a medium supplemented with 10% fetal calf serum and incubated at 37°C. All initial cultures were gassed with 5% CO_2. Stock cultures were routinely replenished with fresh medium every two days and transferred about every five days. To avoid the possibility of contaminations all work involving open cultures was done in a tissue culture hood. Growth rate data were obtained through the use of two techniques: enumeration of cell populations with a Coulter counter and the metaphase index. Chromosome (metaphase) spreads were prepared by a colcemid-hypotonic technique and stained with Giemsa. The metaphases were studied with oil immersion microscopy for structural and numerical aberrations. The reversible-temporary or irreversible-permanent effects of the two compounds were analyzed by the use of metaphase indices and chromosome preparations.

On the basis of the 72-hour growth rate studies in which cells were exposed to various concentrations of ethyl carbamate or methoxyethyl carbamate, the findings revealed that the effects of the two compounds could be grouped into categories of: stimulation or retardation of growth rates or lethal effects. Both compounds stimulated the growth rates at concentrations of 30 and 300 μg/ml. At 3000 μg/ml the effects of the two compounds differed greatly, ethyl carbamate retarded growth, but methoxyethyl carbamate stimulated growth. Both compounds at 6000 and 9000 μg/ml retarded the growth rates of these cells. The degree of retardation was inversely proportional to the dosage level of the two compounds. At 12,000 and 15,000 μg/ml, methoxyethyl carbamate continued to cause reduced growth rates which were inversely proportional to the dosage level tested. Ethyl carbamate at these concentrations produced effects of a direct proportional relationship between the concentration tested and the percentage of lethality. Both compounds at 30,000 μg/ml were found to be completely lethal to Chinese hamster fibroblasts.

On the basis of the results obtained from the 72-hour growth rate studies it was concluded that: a) the effects of the two compounds were dosage dependent; both compounds stimulated growth in the lower concentrations and retarded growth in the higher concentrations tested; b) for complete lethality, dosages in excess of 15,000 μg/ml were needed for both compounds; c) ethyl carbamate was more toxic to these cells than was methoxyethyl carbamate; and d) methoxyethyl carbamate was a more effective compound than ethyl carbamate in both the stimulation and retardation of growth.

The results of the comparisons of the effects of ethyl carbamate

and methoxyethyl carbamate at 300 and 3000 µg/ml on the doubling time of cell populations were obtained from two separate, but similar, 48-hour experiments. In both experiments, slight differences were detected in the amounts of time required for the treated populations to double for the individual doubling periods. However, in each experiment, when the mean doubling time for each of the experimental populations was compared with the mean doubling time for its respective control, no significant difference was found. However, from these studies, results with reference to growth rates were comparable to those found in the 72-hour growth rate experiments previously discussed. Based on the results of these experiments it was concluded that neither ethyl carbamate nor methoxyethyl carbamate at 300 or 3000 µg/ml significantly affected the average doubling time of these cells.

In order to study the effects of ethyl carbamate and methoxyethyl carbamate at sublethal concentrations (300 and 3000 µg/ml) in causing chromosomal aberrations cells were exposed to these compounds for 6, 12, 24, 48 and 72 hours. The results of this experiment clearly indicated that both compounds at the two tested dosage levels induced chromosomal breaks in Chinese hamster fibroblasts. Ethyl carbamate at 300 and 3000 µg/ml produced statistically significant numbers of cells with chromosomal breaks as early as 6 hours after initial exposure and continued to show statistically significant effects for the remaining test periods. The same was true for methoxyethyl carbamate at 3000 µg/ml. Methoxyethyl carbamate at 300 µg/ml did not produce a statistically significant number of cells with chromosomal breaks until after 24 hours of exposure. The compound at this dosage level, however, continued to produce statistically significant numbers of

cells with chromosome breaks for the remaining test periods. Further, the compound after 48 hours of exposure produced cells with multiple breaks, fragmented or pulverized chromosomes.

Both compounds at the two levels tested caused mostly CHR breaks which occurred more frequently in the LM and ACRO chromosomes. SM chromosomes were very rarely affected by either of the compounds. ISO breaks were caused by the action of both compounds and occurred more frequently in the LM chromosomes than in the ACRO chromosomes. ISO breaks were not found in the SM chromosomes. From the results of the chromosome breakage data, it was concluded that: a) the effects of ethyl carbamate at 300 and 3000 µg/ml and methoxyethyl carbamate at 3000 µg/ml were non-delayed or non-cumulative; b) the effects of methoxyethyl carbamate at 300 µg/ml were delayed or cumulative; and c) the two levels of the compounds showed specificity for attacking the LM and ACRO chromosomes rather than the SM chromosomes.

From the data on numerical aberrations, ethyl carbamate at 300 µg/ml showed the most consistent trend in altering the chromosome number of the cultured cells. To a lesser extent, ethyl carbamate at 3000 µg/ml was effective in causing deviations in the distribution of the chromosome number of the cells. From the data on chromosome numbers, it was concluded that ethyl carbamate at 300 µg/ml and to a lesser degree at 3000 µg/ml altered the chromosome number of the cells by reducing the frequency of approximately "diploid" metaphases and increasing the frequency of "triploid and tetraploid" metaphases. Further it was concluded that neither of the levels of methoxyethyl carbamate significantly or rather consistently affected the chromosome number of the cells.

The results of this experiment in which ethyl carbamate and methoxyethyl carbamate at 300 µg/ml were tested and compared with reference to effects being either reversible-temporary or irreversible-permanent showed differences between the effects of the two compounds. In this experiment three parameters of the effects of the compounds were measured: chromosome breakage, chromosomal numerical aberrations and growth rate as determined by the metaphase index method. Cell populations were exposed to medium containing either ethyl carbamate or methoxyethyl carbamate for 36 hours (treatment period I) then returned to culture in normal medium lacking either of the compounds for 36 additional hours (treatment period II).

After the initial 36-hour exposure (period I) both ethyl carbamate and methoxyethyl carbamate caused the production of statistically significant numbers of cells with chromosome breaks when compared with the untreated control. Within treatment period I, only the effects ethyl carbamate significantly affected the distribution of the chromosome number of the cells. Methoxyethyl carbamate was ineffective in producing numerical chromosomal changes. The effects of ethyl carbamate in this experiment with reference to chromosome number were a reduction in the number of the approximately "diploid" metaphases and an increase in the number of "triploid and tetraploid" metaphases. Within treatment period I, only cells treated with ethyl carbamate showed a statistically significant deviation in the number of cells in colcemid metaphase and the compound caused an increase in the metaphase index. The effects of methoxyethyl carbamate on the growth rate as indicated by the metaphase index did not differ significantly from the control.

Within treatment period II, ethyl carbamate continued to produce

significant numbers of cells with chromosomal breaks, but the same was not true for methoxyethyl carbamate. Neither of the two compounds significantly altered the distribution of the chromosome number of the cells within treatment period II. The number of cells in colcemid metaphase as attested by the metaphase index data for both compounds was not significantly different from the controls. Therefore, it was concluded from the data of this experiment that the effects of ethyl carbamate on chromosomal breakage were irreversible-permanent, but its effects on numerical aberrations and growth rates were reversible-temporary. It was further concluded that the effects of methoxyethyl carbamate on chromosomal breakage were dependent upon its presence and were thus temporary-reversible. Methoxyethyl carbamate does not produce significant effects on altering the chromosome number of the cells or the metaphase index.

Assessment of all the data collected in this research showed that there were certain similarities and dissimilarities between the effects of ethyl carbamate and methoxyethyl carbamate on the growth and chromosomes of Chinese hamster fibroblasts. The use of the effects of ethyl carbamate, a reported carcinogen in vivo, as a model for the in vitro testing of methoxyethyl carbamate (a structurally similar compound) as a probable carcinogen were inconclusive from the results of this investigation. It was felt that the similarities of the effects which existed between the two compounds were not consistent enough for a valid conclusion that methoxyethyl carbamate was a carcinogen. However methoxyethyl carbamate at 30, 300 and 3000 μg/ml did cause statistically significant increases in the growth rates of cell populations and at the latter two dosage levels caused the production of statistically

significant numbers of cells with chromosomal breaks. Increased growth rates and the production of chromosomal breaks are some of the characteristics often used by researchers in testing chemicals for carcinogenic properties. The reported and most conclusive evidence for testing for carcinogenicity of compounds in an in vitro system is the injection of suspected "transformed" cells into immunologically computible hosts and the subsequent development of tumors in these animals. This approach, in addition to direct injections of a compound like methoxyethyl carbamate into animals in testing for carcigogenic effects seems to be a much needed avenue for research. Based on the comparisons made between the effects of the two compounds, in this study, it was concluded that methoxyethyl carbamate was certainly a mutagenic compound, if not carcinogenic. However further studies for testing its possible carcinogenicity are needed.

LITERATURE CITED

American Type Culture Collection. 1964. Registry of animal cell lines certified by the cell culture collection committee. American Type Culture Collection Cell Repository, Rockville, Maryland. CCL 16.

Bastrup-Madsen, P. 1949. Action of mitotic poisons in vitro. I. Effect of urethane on division of fibroblasts. Acta. Path. Microbiol. Scand. 26:93-112 (Biol. Abstr. 23(2):17813).

Battaglia, E. 1949. On the action of ethyl carbamate (urethan) and cyclohexyl-carbamate. Caryologia 1:229-247.

Battle, H. I. and K. K. Hisaoka. 1952. Effects of ethyl carbamate (urethan) on the early development of the teleost Brachydanio rerio. Cancer Res. 12:334-340.

Berman, L. and A. R. Axelrod. 1948. Effect of urethane on malignant diseases. Clinical, hematologic and histologic observatins on patients with carcinoma, leukemia and related diseases. Am. J. Clin. Path. 18:104-129.

Boyland, E. and P. C. Koller. 1954. Effects of urethane on mitosis in the Walker rat carcinoma. Brit. J. Cancer 8:667-684.

Bucher, O. 1949a. The influence of ethyl carbamate on cell division in tissue culture in vitro. Helvet. physiol. pharm. acta 7:37-54.

Bucher, O. 1949b. The action of ethylurethan on the course and speed of division in tissue cultures. Schweiz. Med. Wochenschr. 79:487. (Biol. Abstr. 25(1):98).

Burger, G. 1968. The influence of ethyl urethane and cyclophosphamide on reproduction and oxygen consumption of Leptospira semaranga (Veldrat S 173). Zentralbl. Bakteriol. Parasitenk. Infektionskrank. Hyg. Abt. I. Orig. 207:365-376. (Biol Abstr. 51(1)21413).

Cancer Chemotherapy National Service Center. 1962. Cell Culture technical procedures. Cancer Chemotherapy Report No. 25. p. 57-58.

Colnaghi, M. I. 1969. Chromosomal changes associated with urethane leukemogenesis in mice. Int. J. Cancer 4:327-333.

Creskoff, A. J., T. Fritz-Hugh and J. W. Frost. 1948. Urethane therapy in leukemia. Blood 3:896-910.

Deufel, J. 1951. Influence of chemicals and X-radiation on mitosis of V. faba. Chromosoma 4:239-272.

_____. 1952. Chromosoma 4:611. In J. J. Biesele. 1957. Mitotic poisons and the cancer problem. Elsevier Pub. Co. New York. p. 81.

Driessens, J. 1952. Inhibitory effect of ethyl urethan on cultures of fibroblasts from the embryonic chick heart. C. Rend. Soc. Biol. 146:1112-1114.

Dustin, P. 1947. The cytological action of ethyl carbamate (urethane) and other carbamic esters in normal and leukemic mice, and in rabbits. Birt. J. Cancer 1:48-59.

_____. 1949. Urethane and its combination with X-ray therapy in human leukemias. Rev. Belge Path. 19:115-174 (Abstr. World Med. 6(1) 2445).

Ford, D. K. and G. Yerganian. 1958. Observations on the chromosomes of Chinese hamster cells in tissue culture. J. Nat. Cancer Inst. 21:393-425.

Geiersbach, U. 1939. Uber den Einfluss der Narkose Urethan) auf Gewebekultren Archiv. Exp. Zellforsch. bes. Gewenbezucht. 23: 210-219 (Biol. Abstr. 14(1):930).

Globerson, A. and R. Auerbach. 1965. In vitro studies on thymus and lung differentiation following urethan treatment. Wistar Inst. Symp. Monogr. 4:3-19.

Goodman, M. J. and H. P. Lewis. 1946. Urethan in leukemia. J. Am. Med. Assn. 132:1105-1111.

Green, J. W. Jr. and C. C. Lushbaugh. 1949. Histopathologic Study of the mode of inhibition of cellular proliferation by urethane: effect of urethane on Walker rat carcinoma 256. Cancer Res. 9: 199-209.

Guyer, M. F. and P. E. Clause. 1947a. Tumor of the lung in rats following injections of methane (ethyl carbamate). Cancer Res. 7:342-345.

Guyer, J. F. and P. E. Clause. 1947b. Effects of urethane (ethyl carbamate) on mitosis. Proc. Soc. Exp. Biol. Med. 64:3-5.

Haddow, A. and W. A. Sexton. 1946. Influence of carbamic esters (urethanes) on experimental tumors. Nature (Lond.) 157:500-503.

Harbel, K. 1968. The biology of viral carcinogenesis. Cancer Res. 28:1825-1831.

Henshaw, P. S. and H. L. Meyer. 1944. Minimal numbers of anesthetic treatments with urethane required to induce pulmonary tumors. J. Nat. Cancer Inst. 4:523-525.

_____. 1945. Further studies on urethane induced pulmonary tumors. J. Nat. Cancer Inst. 5:415-417.

Hirschboek, J. S., M. C. F. Lindert, J. Chase and T. L. Calvy. 1948. Effects of urethane in the treatment of leukemia and metastic tumors. J. Am. Med. Assn. 136:9 0-94.

Hogreffe, G. and E. Pedersen. 1950. Urethan treatment of leukemia in mice. Acta. Path. Microbiol. Scand. 27:3-8.

Hohl, K. 1947. Die Beeinflussing der Mitose durch Urethane (The influence of urethane on mitosis). Exprerientia. 3:109-110.

Hsu, T. C. and K. Benirschke. 1967. An atlas of mammalian chromosomes. Volume I, Folio 13. Springer-Verlag, New York.

Huggins, C., S. T. Yu and R. Jones. 1947. Inhibitory effects of ethyl carbamate on prostatic cancer. Science 106:147-148.

Jaffe, W. G. 1947. Carcinogenic action of ethyl urethane on rats. Cancen Res. 7:107-112.

Johnson, F. H. 1942. Mechanism of p-aminobenzoic action and the parallel effects of ethyl carbamate (urethane). Science 95: 104-105.

Kirschbaum, A. and C. S. Lu. 1947. Effect of urethane on maturation of leukocytes of mouse myelogenous leukemia. Proc. Soc. Exp. Biol. Med. 65:62-63.

Koller, P. C. 1954. Chromosome breakage. Prog. Biophys. Biophys. Chem. 4:195-243.

Larsen, C. D. 1947a. Pulmonary-tumor production by transplacental exposure to urethane. J. Nat. Cancer Inst. 8:63-70.

Larsen, C. D. 1947b. Evaluation of the carcinogenicity of a series of esters of carbamic acid. J. Nat. Cancer Inst. 8:63-70.

Larsen, C. D. 1948. Pulmonary-tumor induction with alkylated urethans. J. Nat. Cancer Inst. 9:35-37.

Larsen, C. D. and W. E. Heston. 1945. Induction of pulmonary tumor in mice by anesthetic agents. Abstr. Proc. 36th Ann. Meet. Am. Assoc. Cancer Res. Cancer Res. 5:592.

Lasnitzki, I. 1949. Some effects of urethane on the growth and mitosis of normal and malignant cells *in vitro*. Brit. J. Cancer 3:501-509.

Moeschlin, S. and A. Bodmer. 1950. Agranulocytosis and panmyelopathy experimentally produced by urethane in the cat. Sang. 21:515-516. (Abst. World Med. 8(2)2349).

Morgans, L. F. 1968. The effects of urethan on fish epithelial and fibroblast cells in vitro. Ph.D. Thesis. Oklahoma State University.

Nettleship, A. and P. S. Henshaw. 1943. Induction of pulmonary tumors in mice with ethyl carbamate (urethane). J. Nat. Cancer Inst. 4:309-319.

Oberman, B. 1965. Experimental melanoma in hamsters. In: Structure and control of the melanocyte, 25-29 May, 1965. Sofia, Bulg. Springer-Verlay, Inc., New York. 268-273. (Biol. Abst. 48(3) 44042).

Oehlkers, F. 1943. Die Auslosung von Chromosomenmutationen in der Meiosis durch Einwirkung von Chemikalien. Z.I.A.V. 81:313-341. In J. J. Biesele. 1957. Mitotic poisons and the cancer problem. Elsevier Pub. Co. New York. p. 81.

Oehlkers, F. and G. Linnert. 1949. Further experiments on the mode of action of chemicals in the production of chromosome mutations. Z.I.A.V. 83:136-156 (Biol. Abst. 25(3)26248).

Osgood, E. E. 1948. The effect of urethane on the nuclear morphology of cells of the granulocyte series as observed in marrow cultures and leukemic blood. Blood 3:911-917.

Patterson, E. and A. Haddow. 1946. Leukemia treated with urethane compared with deep X-ray therapy. Lancet 250:667-682.

Patterson, E. and M. V. Thompson. 1949. Effect of urethan combined with X-rays on chick fibroblasts. Nature (Lond.) 163:563.

Pogosyants, E. E., G. M. Platonova, E. N. Tolkacheva, and L. F. Ganzenko. 1968. The effect of urethane on the chromosomes of mammalian cells in vitro. Genetika 4:60-70 (Chem. Abst. 70(1)298Y).

Priest, J. H. 1969. Cytogenetics. Lea and Febiger, Philadelphia. 233 p.

Rao, S. R. 1961. The action of urethane on chromosomes of the grasshopper, Poecilocera picta (Fabr.). Mem. Indian. Bot. Soc. 3: 99-101 (Biol. Abst. 46(6)73591).

Rosin, A. 1951. Effect of urethane (ethyl carbamate) on the mitotic activity in the bone marrow of normal mice. Blood 6:652-66.

Rothfels, K. H. and L. Siminovitch. 1958. An air drying technique for flattening chromosomes in mammalian cells grown in vitro. Stain Technol. 33:73-77.

Skipper, H. E. and C. E. Bryan. 1948. Carbamates in the chemotherapy of leukemia. III. The relationship between chemical structure and anti-leukemic action of a series of urethan derivatives. J. Nat. Cancer Inst. 9:391-397.

Skipper, H. E., C. E. Bryan, W. H. Riser, Jr., M. Welty and A. Stelzenmuller. 1948. Carbamates in the chemotherapy of leukemia. II. The relationship between chemical structure, leukopenic action and acute toxicity of a group of urethan derivatives. J. Nat. Cancer Inst. 9:77-88.

Tannebaum, A. and C. Maltoni. 1962. Neoplastic response of various tissues to the administration of urethane. Cancer Res. 22:1105-1112.

Tannebaum, A. and H. Silvertone. 1958. Urethan (ethyl carbamate) as a multipotential carcinogen. Cancer Res. 18:1225-1231.

Tjio, J. H. and A. Levan. 1956. The chromosome number of man. Hereditas 42:1-6.

Toplin, I. 1959. A tissue culture cytotoxicity test for large-scale cancer chemotherapy screening. Cancer Res. 19:959-965.

Union Carbide Corporation. 1967. Chemicals for durable press and easy care finishes. Union Carbide Corporation, New York. Booklet.

Watkins, C. H., T. Cooper and H. Z. Griffin. 1948. The use of urethan (ethyl carbamate) in the treatment of leukemia. A preliminary report. Blood 3:892-895.

Wakonig-Vaartaja, R. 1964. The effect of urethan on mitotic cells of mice of different ages and strains. Australian J. Exp. Biol. Med. Sci. 42:165-172 (Biol. Abst. 45(7)89449).

VITA

Nathan Howard Cook

Candidate for the Degree of

Doctor of Philosophy

Thesis: AN IN VITRO COMPARISON OF THE EFFECTS OF ETHYL CARBAMATE AND METHOXYETHYL CARBAMATE ON THE GROWTH AND CHROMOSOMES OF CHINESE HAMSTER FIBROBLASTS

Major Field: Zoology

Biographical:

Personal Data: Born in Winston-Salem, North Carolina, April 26, 1939, the son of Mr. and Mrs. M. T. Farrow.

Education: Graduated from Atkins High School, Winston-Salem, North Carolina, in June, 1957; received the Bachelor of Science degree from North Carolina Central University, Durham, North Carolina, with a major in Biology in June, 1961; received the Master of Arts degree from North Carolina Central University, Durham, North Carolina, with a major in Education and a minor in Biology, June, 1963; studied further at the University of North Carolina at Greensboro, Greensboro, North Carolina, summer, 1964 and North Carolina State Univeristy, Raleigh, North Carolina, summer, 1965; completed requirements for the Doctor of Philosophy degree at Oklahoma State University in May, 1972, with a major in Zoology. Study for the doctorate degree was supported by the Ford Foundation, 1969-1971 and Barber-Scotia College 1968-1969.

Professional Experience: Employed as an assistant professor of Biology at Barber-Scotia College from 1962-1968; appointed as a visiting instructor in Audio-Visual Education at North Carolina Central University, summer 1967, served as graduate teaching assistant in Zoology at Oklahoma State University, 1968-1969.

Professional Organizations: National Institute of Science, Tissue Culture Association and American Association for the Advancement of Science.