IN VITRO EFFECTS OF SODIUM AND CALCIUM CYCLAMATES, CYCLOHEXYLAMINE AND SUCROSE ON GROWTH RATE AND CHROMOSOMES OF CHINESE HAMSTER FIBROBLASTS

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CHAPTER I

INTRODUCTION AND HISTORICAL REVIEW

No food additive has aroused more controversy and general public and scientific concern than the cyclamates. The cyclamates, used as food and drink sweeteners, are the sodium and calcium salts of cyclohexylsulphamic (cyclamic) acid. They have a sweetening power some 30 times that of sugar but do not have the same technological properties as, or the nutritional values of sugar. Cyclamates have no nutritive value whatever and unlike saccharin they leave no bitter aftertaste (Chedd, 1968).

Much research on the safety of cyclamates has been carried out since its accidental discovery by Michael Sveda in 1937 (Beck, 1969). Abbott Laboratory scientists learned of this discovery and recognized its potential as a dietary aid for diabetics and chronically obese people. After more than six years of research by standard toxicological and biological methods Abbott Laboratories of North Chicago, Illinois, marketed the first commercial cyclamate product, a 125 mg sodium cyclamate tablet sold through drug stores with approval by the U.S. Food and Drug Administration (FDA).

All research through the 1950's indicated that cyclamate was safe for human consumption. Audrieth and Sveda (1944) fed cyclamate to rabbits, then examined the urine and feces and were able to recover 80 - 90% of the cyclamic acid. It was concluded that this substance

was not utilized by the rabbits and was not being degraded into toxic products in the digestive tract. In 1951 Taylor, Richards and Davin (1951) supported the 1944 findings of Audrieth and Sveda. Richards, Taylor, O'Brien and Duescher (1951) studied the effects of calcium cyclamate on cats, rats and dogs and found no adverse histological or physiological effects except for occasional stool softening. They also observed no *in vitro* effects while studying isolated rabbit intestine and frog heart. The findings of Richards et al. were substantiated by studies of Fitzhugh and Nelson (1950) and Fitzhugh, Nelson and Frawley (1951). They reported that feeding rats saccharin and sodium cyclohexyl sulfamate (cyclamate) for over two years at concentrations up to 1% of these compounds produced no toxicological effects. Concentrations of 5% produced only slightly toxic effects. Schoenberger, Rix, Sakamoto and Kark (1953) reported that daily administration of 5 gms of calcium cyclamate to human subjects for 7.5 months caused no other effects than changes in bulk of the stools.

In 1958, when the Federal Food Additive Amendment was enacted, cyclamate was placed on the "generally recognized as safe list" along with salt, pepper, cinnamon, citric acid and many other food additives. Following extensive research and testing of human subjects the Food and Drug Administration recommended that intakes of 50 mg/kg body weight per day were safe. This concentration is equivalent to about 3.5 grams of pure cyclamate taken in by an adult or to the amount found in 10 bottles of diet cola (Taylor, 1969). This action was given express approval by the Food Protection Committee (1959). Research on artificial sweeteners continued. Hwang (1966) showed that cyclamate salts exert a hypertonic effect in the alimentary

tracts of man, dogs and monkeys causing water retention. He indicated that stool softening appeared to be the result of hypertonic inhibition of dehydration of feces and was not a manifestation of a true pharmacodynamic action. Fregley and Kier (1966) saw no water balance changes following cyclamate administration to rats with extensive renal disease.

Derse and Daun (1966) analysed by gas chromatography the excreta (urine and feces) of rats fed known amounts of cyclamate and were able to recover only one-quarter of it. This led to the disquieting conclusion that three-quarters of the ingested cyclamate was being stored in the body or being converted to something unrecognizable. Later however, Wiegand (1969) explained that the method used by Derse and Daun in feeding the rats was such that the rats avoided the very sweet (5 - 10%) powdered cyclamate mixed in the food. Wiegand indicated that Derse and Daun did not analyse the remaining food not eaten by the rats. Wiegand's contentions were supported by research of Murray, Wells, Kohn and Miller (1953) and Grinstead (1960) on rats and baby pigs, respectively. They found that these animals avoided sweetened foods when possible. They indicated that when given choices the animals chose the unsweetened food and left the cyclamate or artificially sweetened food.

The steady progress in the establishment of cyclamate's safety-in-use suffered a setback with the discovery by Kojima and Ichibagase (1966) that cyclamates are converted to cyclohexylamine in some humans. They found 0.7% cyclohexylamine in the urine of a human volunteer. Cyclohexylamine, a breakdown product or metabolite of cyclamate, is considered toxic and does produce pharmacologic effects.

Since 1966 many studies have been reported where concentrations of cyclohexylamine up to and greater than 0.8% have been found in humans fed from 1 - 3 gm cyclamate/day (Leahy, Wakefield and Taylor, 1967; Leahy, Taylor and Rudd, 1967; Berryman, 1968; Wills, 1968; and Sonders and Wiegand, 1968). Conversion of cyclamate to cyclohexylamine has also been reported in other animals: dogs (Golberg, Parekh, Patti and Soike, 1969); rats (Oser, Carson, Vogin and Sonders, 1968); dogs and rats (Taylor, Richards, Wiegand and Weinberg, 1968).

Sonders, Wiegand and Netwal (1967) identified a converter rat and fed it cyclamate labelled with radioactive carbon. The tagged cyclamate passed through the intestine and about 47% of it was converted to cyclohexylamine. When tagged cyclamate was given intravenously, thus not passing through the intestine, almost no cyclohexylamine was observed. They attributed the 47% conversion to microorganisms in the intestine. Further, when an antibiotic that kills intestinal bacteria was given to the rats, the conversion dropped from 47% to almost undetectable levels. Taylor, Richards, Wiegand and Weinberg (1958) reported that only some animals and humans (approximately 10 - 15% of the population) are able to convert cyclamate to cyclohexylamine. They contend that the conversion takes place in the intestine by bacteria and that only organisms with these intestinal bacteria are converters. Hence, neither substance is absorbed from the intestine into the blood. Contrary to the findings of Sonders et al. (1968) and Richards et al. (1968), Davis, Adler and Ospahl (1969) gave 11 human volunteers 1 - 3 gms of sodium cyclamate daily and found that all 11 excreted cyclohexylamine in the urine and

and feces (0.04 - 154 mg/day). They also incubated cyclamate with fresh human feces and found that it was not converted anaerobically to metabolites.

Analysis of cyclohexylamine in food and drink has been enhanced by the development of newer and more sensitive methods of detection. One method devised by Bradford and Weston (1969) utilizes chloroform for extraction. The chloroform complexes with cyclohexylamine to form a compound that can be measured absorptiometrically. Amounts as low as 10 ppm can be determined. Another method developed by Howard, Fazio, Klineck and White (1969) can detect at levels down to 0.2 ppm. Woidich, Gnaver and Galinovsky (1969) developed a method of extracting saccharin and cyclamate from food stuffs for analysis by gas chromatography. With this method they detected quantities of sweeteners at less than 6 mg/liter from fruit juices.

Many studies and types of studies have been conducted on various species of animals. Some studies have indicated ill-effects while others have counteracted with no effects using similar experimental procedures and animals. Effects on embryogenesis, teratogenicity, skeletal development, decreases in growth and food utilization, or reproductive functions in various species have been claimed by: Tanaka (1964), mouse embryos; Nees and Derse (1965 and 1967), rats; Ghiani and Muratori (1969), chickens; and Becker and Gibson (1970), primigravid Swiss mice. No harmful or deleterious effects on the above processes have been reported by the following investigators using the listed animals: Kennedy, Sonders, Weinberg, Arnold and Keplinger (1969), rabbits; Oser, Carson and Vogin (1968), rats;

Fritz and Hess (1968), rats; Dalderup and Visser (1969), rats; Weinberg and Horrington (1968), rats; Taylor, Richards, Wiegand and Weinberg (1968), rats and dogs; and Khera, Stoltz, Gunner, Lyon and Grice (1971).

Effects on the heart, smooth muscle tissue and physiological stress leading to death have been reported by: Rosenblum and Rosenblum (1968), cats; Rosenblum and Rosenblum (1968), guinea pig intestine; Lee and Dixon (1969), Swiss-Webster mice, respectively. Myocardial lesions accompanied by coronary schlerosis and soft calcification have been reported in hamsters from oral administration of calcium cyclamate by Bajusz (1969).

Studies of acute and chronic effects by the following investigators indicated no significant adverse physiological or behavioral effects with the exception of stool softening: Fancher, Palazzolo et al. (1968), dogs; Golberg et al. (1969), dogs; Roe, Levy and Carter (1970), mice; and Taylor et al. (1968), dogs and rats.

Stein, Serrone and Coulston (1967) using electron microscopy, observed diffuse mild vesiculation of the endoplasmic reticulum associated with vacuolization in the liver and kidneys of monkeys fed high concentrations (8 gm/kg) of sodium cyclamate.

Volm and Goerttler (1965) using in vitro tests found that sodium cyclamate had no effects on DNA, RNA and protein synthesis at 10^{-2} molar in Tetrahymena pyriformis, L-cells and liver explants. It should be noted that in nearly all of the studies reported the concentrations used were far above the normal intake capacity or levels of the animals used.

On October 18, 1969, Robert H. Finch, then Secretary of U.S. Health, Education and Welfare and Jesse L. Steinfeld, Deputy Assistant Secretary for U.S. Health and Scientific Affairs announced at a press conference that the cyclamates were being removed from the list of substances "generally recognized as safe" for use in foods. They also announced an order halting the use of cyclamates in the production of general purpose foods. A phase-out schedule for products containing cyclamate was also issued. They reported that the decision to ban cyclamates from the public market came from a review of new scientific evidence by officials of the National Academy of Science and the National Cancer Institute. The evidence was that researchers at the University of Wisconsin, Madison, Wisconsin, and at Abbott Laboratories found that injecting or feeding rats high concentrations of cyclohexylamine or a mixture of sodium cyclamate and saccharin (10:1) caused the formation of urinary bladder tumors (Price et al., 1970). Both Finch and Steinfeld indicated that there was no cause for alarm because no evidence had shown that cyclamates caused cancer in humans. Secretary Finch indicated that in issuing the ban he acted under the provisions of the law because "it is imperative to follow prudent course in all matters concerning public health." The law he referred to is the Delaney Amendment to the Food, Drug and Cosmetics Act of 1958 which states that any food additive must be removed from the market if it has been shown to cause cancer when fed to humans or animals. Following or concurrent with the U.S. ban, many other countries including Canada, Britain, France and Sweden ordered a withdrawal of cyclamates from the market.

Although the U.S. ban called for a removal of cyclamate from food products, it did not exempt the availability to persons whose health depends upon artificial sweeteners for medical care for such conditions as diabetes or obesity. Since the Delaney Amendment concerns food and not drugs, cyclamate was then proposed by a Medical Advisory Group on Cyclamates, to be termed as a drug. However, since 1962 the FDA has required proof of efficacy before any new drug can be offered for human consumption. No such proof had been presented for cyclamates and in fact there was reason to believe that they were not effective (Bazell, 1970). In August, 1970, after much deliberation and study of data available, the Medical Advisory Group on Cyclamates suggested to HEW that cyclamates be disapproved for human consumption even as drugs. On August 14, 1970 the sale of all cyclamates was banned by HEW.

Much controversy still exists concerning the status of cyclamate safety especially as to its possible ability to cause cancer. The question of its harmfulness to man is still unanswered since urinary bladder tumors or other lesions similar to those found in animal studies have not been reported from human studies. Newsletters, notes to the editor, brief notes to scientific and general publications have been written by many people. Some agree with the ban whereas others disagree. Many have pointed out that the cyclamates have been in use for over 20 years with no recorded deleterious effect and that the immediate ban was an overreaction. Beaconsfield (1969) indicated that many substances, including salt, when administered at some of the concentrations reported, may cause effects similar to those found with cyclamate. Yudkin (1969) feels that excess sugar consumption

may be more harmful than cyclamates. Chedd (1968), Berglund (1969), Anderson and Brady (1969) are just a few of the many people who sanction the ban on cyclamates.

Chedd (1968) indicated that the fact that no ill-effects have been detected in the population at large after years of usage is not necessarily proof of cyclamate safety. He also pointed out that a "low incidence" effect may go undetected for some considerable time as is apparently the case, for example, with thrombosis and the oral contraceptive pill. Considering Chedd's point of view the greatest challenge to safety of cyclamates may come from another direction.

Newer techniques, which are concerned with the detection of effects on genetic material, chromosomes, have now been developed for studying and assessing chemical and drug safety. Some types of chromosomal changes, such as breaks, are commonly found in a small percentage of body cells. Geneticists and cytogeneticists are concerned if the number of such chromosomal changes exceeds the normal. The concern derives from the fact that chromosomal damage may indicate genetic changes which may be passed on to the next generation as mutations, and most mutations are considered harmful (Dick, 1969).

Food additives or components have been known to cause chromosomal aberrations in plants since Kihlman and Levan (1949) discovered that caffeine and theophylline induced structural chromosomal changes in onion root tips. It was probably the discovery of Ostertag (1965, 1966) that caffeine and theophylline induced breakage of Hela cell and leukocyte chromosomes, followed by the discovery of Cohen, Marionello and Back (1967) that lysergic acid diethylamide (LSD)

caused breaks in chromosomes of human leukocytes, that prompted intensive studies on the cytogenetic effects of food additives. Cohen's findings were substantiated by research of Sparks, Melnyx and Bozetti (1968) and Irwin and Egozcue (1967). Sax and Sax (1968) discovered that Sucaryl (sodium cyclamate) at relatively high concentration caused a significant amount of chromosome breakage in onion root tips as compared to control root tips not exposed to Sucaryl.

The first report that cyclamate or its metabolites cause chromosomal damage in animals was given by Legator, Palmer, Green and Petersen at the Seventh Conference on Mammalian Cytology and Somatic Cell Genetics held at Gatlinburg, Tennessee, October 23-26, 1968. This report was subsequently published in Science magazine, September 12, 1969. They found that injecting rats with cyclohexylamine caused a significant increase in the number of chromosome breaks in rat bone marrow and spermatogonial cells. Since that time two other groups of investigators have found evidence that cyclamates cause chromosome breaks in human leukocytes and cultured human cells, in vitro (Stone, Lamson, Chang and Pickering, 1969 and Stoltz, Khera, Bendall and Gunner, 1970). In contrast to these three studies, Dick (1969) reported that Dr. J. H. Wills at Albany Medical College, Albany, New York, gave doses of cyclamate as large as 16 gm per day to 24 human volunteers and found no more chromosome breaks than with a control group given no cyclamate. She noted that in this study 17 of the 24 volunteers were cyclamate converters.

Very little work has been published on the effects of cyclamates on cells and chromosomes in vivo or in vitro. Effects on growth rate

and morphology of cells after exposure to cyclamates have not been reported to the author's knowledge. All of the published studies on chromosome breakage have come from short term exposures of five days or less. Although structural aberrations have been reported, no reports of numerical aberrations are known by the author.

The objectives of this investigation were to determine: (1) the effects of different concentrations of sodium cyclamate, calcium cyclamate, cyclohexylamine and sucrose on growth rate, chromosome breakage and chromosome number after short term exposures; (2) the effects of long term exposures to "low" concentrations of sodium cyclamate on the above three parameters and (3) whether any observed changes were permanent, temporary or latent, in Chinese hamster fibroblasts *in vitro*.

CHAPTER II

MATERIALS AND METHODS

Cell Culture Used

Cells of the Don strain of Chinese hamster (Cricetulus griseus) lung, obtained from Grand Island Biological Company (GIBCO), Grand Island, New York were used in this study. This strain has been reported to be diploid, fibroblast-like and of interest to investigators for virus, chromosome and autoradiograph studies. The Don strain was isolated in September, 1962 from a normal eight-month old Chinese hamster by T. C. Hsu (no publication). The modal chromosome number of the strain is 22 with a stable diploid karyotype which agrees with that of the normal hamster. Generation time is approximately 12 hours (American Type Culture Collection, 1964).

The male Chinese hamster has 20 autosomes (eight large metacentric to sub-metacentric, six small metacentric and six small sub-telocentric or acrocentric) and two sex chromosomes (X and Y) which are medium sized sub-metacentric (Hsu and Benirschke, 1964).

Medium Used, Routine Growth, Maintenance of Stock Cultures, Transfers, Glassware, Cell Counts

The culture medium used for growth and maintenance of stock cultures was McCoy's 5A (modified) medium containing the following

antibiotics: potassium penicillin G (40 mg/l), streptomycin sulfate (50 mg/l) and neomycin (100 mg/l), supplemented with 10% fetal calf serum. The medium was obtained from Microbiological Associates (MBA), Bethesda, Maryland, and the serum from Grand Island Biological Company (GIBCO), Grand Island, New York.

All cultures were grown as monolayers, buffered at pH 7.0 and gassed initially with 5% carbon dioxide in air, in screw cap Falcon plastic containers. All plasticware (disposable flasks and pipettes) used and subsequently mentioned was obtained from Falcon Plastics, Division of Bioquest, Los Angeles, California. Stock cultures were maintained in 15 ml medium in T-75 flasks (75 cm^2 surface area for attachment). Test cultures were grown in T-25 flasks (25 cm^2 surface area) in 4 ml of medium. All cultures were incubated at 37°C.

The growth medium of stock cultures was routinely decanted and replaced by fresh medium every two days. Every five days, or whenever growth in the flask became confluent, a transfer was made whereby new flasks were seeded or excess cells discarded. This procedure was accomplished as follows: the growth medium was decanted and cells rinsed twice with Puck's saline A (balanced salt solution, BSS). The BSS was poured off and replaced by a trypsin - EDTA (ethylene-diaminetetraacetic acid) solution (GIBCO) in concentrations of 0,05% and 0.025% respectively. Flasks were then reincubated for 2 - 5 minutes to facilitate the release of cells from their growing substrate and to lessen adhesion between the cells. After the cells became detached the suspension was aspirated with a sterile Pasteur pipette equipped with a rubber bulb, to further break up clumps of cells. This homogeneous suspension was then pipetted into a sterile

12 ml conical screw-cap centrifuge tube and centrifuged at 500 - 850 rpm for five minutes in a Model CL International Clinical Centrifuge (ICC). Pasteur pipettes and centrifuge tubes were coated with a silicone product, Siliclad, to minimize cell adhesion to the glass (Clay-Adams Inc., New York, N.Y.). The supernatant (trypsin - EDTA) was decanted and replaced by growth medium. The cells were then resuspended by gentle aspiration with a Pasteur pipette after which aliquots were placed into new flasks or discarded. The desired volume of medium was placed into flasks, cultures gassed with 5% carbon dioxide, labeled and incubated.

All transfers and work done with open cultures were carried out in a sterile hood equipped with an ultraviolet (U-V) germicidal light source. The U-V light was left on whenever the hood was not in use. All working surfaces of the hood were washed with 70% isopropyl alcohol immediately before use. Rigorous aseptic technique was utilized to avoid bacterial contamination.

Elaborate care was taken in the preservation, storage and cleaning of glassware. Used glassware was placed in covered containers in a 0.5% aqueous solution of Micro-Solv tissue culture detergent (MBA) immediately after use. This was done to prevent materials (especially proteinaceous deposits) from drying on glassware. All glassware was washed in a warm solution of Micro-Solv (0.5%), rinsed in running hot tap water, four rinses in deionized water and two final rinses in double deionized glass distilled water. Glassware was then dried in a dry heat oven at 250^{o}F for 1 - 2 hours, wrapped in aluminum foil or placed in sterilizing bags and sterilized by autoclave for 20 minutes at 121^{o}C and 18 psi. Plastic caps, silicone

stoppers, rubber policemen, Swinney filters and syringes were washed in warm tap water, rinsed in the same manner as glassware, air dried and sterilized.

To estimate the number of cells in a population, the cells were harvested as in the transfer procedure and resuspended in Hanks balanced salt solution. Two-tenths ml of the suspension was then added to a vial containing 19.8 ml of filtered normal saline. After thorough suspension, five counts were taken using a model B Coulter counter (Coulter Electronics, Hialeah, Florida) and averaged. A background count (count taken on 20 ml of filtered saline without cells) was subtracted from the sample count, after which the sample count was multiplied by the sample dilution to give the number of cells per ml. This procedure was used in establishing inoculum number and for making final counts for estimates of cell population growth.

Compounds Used and Preparation of Stock Solutions

The compounds tested were sodium cyclamate ($C_6H_{12}NNaO_3S$, mol wt, 201.22), calcium cyclamate ($C_{12}H_{22}CaN_2O_6S_2 \cdot 2H_2O$, mol wt, 432.53), cyclohexylamine ($C_6H_{11}NH_2$, mol wt, 99.17) and sucrose ($C_{12}H_{22}O_{11}$, mol wt, 342). The cyclamates and cyclohexylamine were obtained gratuitously from the Abbott Laboratories, North Chicago, Illinois. Sucrose was taken from the coffee room, fourth floor, Life Science West building.

The principal compound of study was sodium cyclamate; therefore more studies and a closer analysis was made of its effects. All long term exposures were made using this compound.

Stock solutions of the compounds were made up directly into McCoy's 5A medium without serum in the following concentrations: sodium cyclamate and sucrose 1% (10 mg/ml) w/v; calcium cyclamate 0.1% (1 mg/ml) w/v; and cyclohexylamine 1% (10 ml/ml) v/v. The stock solutions were sterilized by filtration through a sterile Millipore filter inside a Swinnex filter holder (Millipore Corporation, Bedford, Mass.) attached to a syringe. The sterile solutions were vialed and stored at 4 - 6°C until needed. Prior to use, the pH was adjusted to approximately 7.0 by use of sterile solutions of 1 N sodium hydroxide or 1 N hydrochloric acid. Test solutions were prepared by diluting aliquots of the stock solution with medium and serum to the desired concentrations. The final concentration of serum in all cases was 10%.

Exposure to Test Compounds and Preparation of Slides for Chromosome Studies

Seventy-two Hour Exposures

To determine the effects on chromosome breakage and chromosome number and to calculate metaphase indices, cells were exposed to 1, 10, 100, 250, 500, and 1000 ug/ml of each test compound for 72 hours. Slides were then prepared for observation and detailed study (concentrations of 250 and 500 ug/ml of sugar were not studied). The following procedure was employed for this operation: after harvesting a sufficient number of cells from stock flasks, equal numbers of cells, suspended in 0.2 ml BSS, were placed into two T-25 flasks for each concentration of the test compound and into two flasks that contained medium not supplemented with test compound (control). All flasks

were then incubated. After 48 hours in culture, medium was changed in each flask. Cultures were then reincubated. Three hours before the 72nd hour, colcemid (GIBCO) at a concentration of 0.1 ug/ml was added to each culture. At the end of the 72 hour period, cells from the two flasks for each concentration were harvested and pooled into one 10 ml centrifuge tube. After centrifugation for five minutes at 850 - 1000 rpm (ICC), the cells were resuspended in a hypotonic solution (0.25 normal strength) of Hanks BSS, incubated for ten minutes in a 37°C water bath and centrifuged. The hypotonic BSS was decanted and a fixative (acetic acid: absolute methyl alcohol, 1:3) added to the cell button gently, and allowed to remain quiescent for 30 minutes. The fixative was decanted and cells resuspended in fresh fixative. Fixative changes and resuspensions were continued until the suspension appeared cloudy and less flocculent.

Microscope slides were prepared for use by soaking in a sulfuric acid-dichromate mixture followed by rinses in hot water, distilled water and 95% ethanol and were stored in 20% ethanol at 4 - 6°C.

After resuspension in fresh fixative, a few drops of the cell suspension were dropped onto the cold slide. The slide was then held 12-15 inches above a low flame to enhance spreading and drying. Slides were allowed to continue air drying at least 24 hours at room temperature, then stained with phosphate-buffered Giemsa for 8-10 minutes. After air drying the slides were mounted with permount. The above procedure of slide preparation is a modification of techniques of Rothfels and Siminovitch (1958); Priest (1969); Tjio and Levan (1956); C. Lovig, Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma; and L. Higgins, Biochemistry Department Oklahoma

State University, Stillwater, Oklahoma (personal communications). Six or eight slides were prepared for each pooled sample. Four of these slides were selected at random for study.

Long Term Exposures to Sodium Cyclamate

Duplicate stock cultures were continuously exposed to sodium cyclamate in concentrations of 1 ug/ml for 97 days; 10 ug/ml for 9, 62 and 124 days; and 100 ug/ml for 3, 7, and 60 days, to determine the effects on chromosome breakage and number after these long periods of exposure. Slides were prepared from two T-25 flasks that had been treated with colcemid as in the 72 hour exposure studies. Controls were prepared at each time interval.

To determine effects on cell division rate, metaphase indices were studied on cells exposed to 10 ug/ml sodium cyclamate for 9, 33, 62, 90 and 124 days.

Analysis of Slides for Chromosome Breaks

A total of 100 well spread metaphases, 25 from each of four slides, were selected at random under low power (100 X) and scored for breaks under oil immersion (970 X) for all cultures exposed to sodium cyclamate and their controls. Only 50 metaphases were scored for the other compounds and their controls. Observations were made with an American Optical series 10 microstar microscope equipped with a 35 mm photomicrographic camera.

The number of cells that exhibited breaks and the total number of breaks observed per 50 or 100 cells were recorded. Abnormalities were scored as breaks only if a clear discontinuity of the chromatid

was visible. Pale staining chromosomal regions were recorded but were not calculated and included in breakage rates and percentages. Both chromatid and isochromatid breaks were scored. The type of chromosome (large metacentric to submetacentric, sub-telocentric or acrocentric, or small metacentric) in which a break occurred was also noted. The distribution of breaks among chromosomes and the frequency of occurrence of break types were analysed for cells exposed to concentrations of sodium cyclamate that caused a statistically significant amount of breakage.

Differences in the number of cells with breaks between exposed and unexposed (controls) cells were analysed by chi-square tests. For analysis two classes were used: (1) the number of cells with breaks/ 50 or 100, and (2) the number of cells without breaks/ 50 or 100, which resulted in one degree of freedom for each test. Example:

$$X^2 = \frac{\text{class 1}}{(\underline{\text{observed - expected}})^2} + \frac{\text{class 2}}{(\underline{\text{observed - expected}})^2},$$
$$\text{expected} \qquad\qquad \text{expected}$$

where observed = exposed, and expected = unexposed (control. The hypothesis being tested was that there were no difference between the exposed and unexposed cultures or cells.

In this nonparametric test, the X^2 value was used to determine the probability of the number of cells with breaks occurring by chance alone. When this probability was less than the chosen level of significance (P .05), the null hypothesis of no difference between exposed and unexposed cells was rejected and a difference between the two groups was considered significant.

Analysis for Changes in Chromosome Number

The number of chromosomes in each of the cells selected for study of chromosome breakage was counted under oil immersion with the aid of a blood cell counter. Following a format similar to that used by Ford and Yerganian (1958), the counted cells were divided into five groups based on the number of chromosomes they possessed. The groups are:

I - cells with less than 20 chromosomes, referred to as less than diploid;

II - cells with between 20-25 chromosomes, called approximately "diploid";

III - cells with between 26-29 chromosomes;

IV - cells with 30-50, referred to as the "triploid and tetraploid groups"; and

V - cells with more than 50 chromosomes, called the "more-than-tetraploid group."

Chi-square tests were used to test for differences in the ratio of cells in the five groups (classes) between the control (unexposed) and experimental (exposed) cultures.

The five groups listed above constituted the five classes giving 4 degrees of freedom for the tests.

Determination of Metaphase Indices

To study the growth rate of cells, the rate of cell division as estimated by the metaphase index was one of the methods used. The metaphase index used was a modification of the mitotic index method

described by Paul (1970). The difference between this method and Paul's method is that colcemid was used to arrest or accumulate cells at the metaphase stage. The only dividing cells counted were metaphases. For analysis, a total of 1000 cells was counted for each test concentration and the control. The number of metaphases and non-metaphases, per 250 cells chosen at random, was counted for each of four slides per concentration. The resulting mean index per 250 cells of each experimental culture was compared with the control index. A nonparametric t test based on range (Snedecor and Cochran, 1967) was used to test for differences between the control and experimental mean number of metaphases. The test is given as:

$$t_w = \frac{\bar{X}_c \quad \bar{X}_e}{\frac{W_c \quad W_e}{2}}$$

where t_w = a t based on range; X_c = the mean number of metaphases of the control; X_e = the mean number of metaphases of experimental cultures; W_c = the range or spread of control metaphases; W_e = the range or spread of experimental metaphases.

Determination of Growth Rate by Cell Counts

72 Hour Exposures

To determine the effects of the test compounds on the growth rate of cells, a modification of an experimental procedure used by the Cancer Chemotherapy National Service Center, CCNSC, (1963) was used.

Several experiments were conducted whereby cells were exposed to the following concentrations of the test compounds for 72 hours: sodium cyclamate at 1, 10, 100, 1000, 5000, 10^4 and 10^5 ug/ml; calcium cyclamate at 1, 10, 100, 500 and 1000 ug/ml; cyclohexylamine at 10, 100 and 1000 ul/ml; and sucrose at 1, 10, 100 and 1000 ug/ml.

In each experiment a number of T-25 flasks were inoculated with equal volumes of a homogenous cell suspension in normal growth medium and incubated for 18-24 hours to allow cells to attach. Six cultures were then selected at random, harvested and counted. An average of the number of cells in the six cultures was taken and used as a baseline (number of cells at zero time). An assumption was made that each of the remaining cultures (not counted) contained this average number of cells. Growth medium was decanted from these cultures. Medium supplemented with each of the different concentrations of the test compound was added to each of two of these cultures. Control medium was placed into one culture for each experimental concentration being studied (for example, if four concentrations were being studied, four controls were used). All cultures were then incubated for 72 hours. After 72 hours incubation, all cultures were observed for attachment, degree of confluency, and changes in metabolism as evident by changes in pH. Changes in pH were detected by color changes of phenol red indicator (pH range 6.8 - 8.4) in the medium. Yellow indicated an acidic condition. This meant that a large number of cells were present and actively metabolizing the substrate to produce large volumes of carbon dioxide. The produced carbon dioxide reacted with water in the medium to produce carbonic acid. Pink-purple indicated an alkaline condition and meant that very little metabolism

had taken place. Orange was intermediate. These observations were used as secondary criteria for assessment of growth. Cultures were then harvested and cell populations counted. The mean number of cells was computed from the two cultures at each concentration. A mean was also taken on control flasks.

To assess the effects of the different concentrations of the test compounds on population growth, the following formula was used:

$$\frac{T - Co}{C - Co} = Y \text{ (growth ratio) or } \frac{\text{Treated}}{\text{Untreated}}$$

where Co = baseline (number of cells at zero time); C = the mean of the controls; and T = the mean of the two experimental cultures.

The growth ratio for experimental concentrations was expressed as percent of growth of the controls. The control has in all cases an assigned growth ratio of 1.000 which is 100%. Any population of cells with a growth ratio greater than 1.000 has a growth rate faster than that of the control. The opposite is true for ratios less than 1.000.

Experiments for determination of growth rate of cells exposed to 10, 100, and 1000 ug/ml of sodium cyclamate were repeated five times. All other concentrations and compounds were studied once.

Analysis of variance employing an F test was used to test the null hypothesis of no differences between concentrations of 0, 10, 100 and 1000 ug/ml. The least significance difference or lsd method according to Steele and Torrie (1960) was used to test for differences between means of control and experimental groups.

Doubling Time of Cell Populations

After plotting the results and averaging the growth ratios of the five experiments on sodium cyclamate, one concentration, 1000 ug/ml, was selected for doubling time studies. Doubling times of cell populations exposed to calcium cyclamate at 1000 ug/ml were also performed to compare the effects of these two compounds. This experiment was performed three times using sodium and two times using calcium cyclamate.

In each experiment, 26 T-25 flasks were seeded with equal aliquots of cells in growth medium and incubated for 24 hours. Six cultures were harvested and counted to establish a baseline cell number. Normal growth and experimental media were added to equal numbers of the remaining 20 cultures. Cultures were then incubated. Duplicate experimental and control cultures were harvested and counted after 12, 18, 24, 36 and 48 hours incubation. The mean number of cells from the duplicate cultures for each time period (both experimental and control) was plotted on semilog graph paper (cell number vs time) and growth curves constructed. To establish doubling times, from each of the growth curves, horizontal lines representing multiples (doubles) of the baseline were constructed on the graphs for each experiment. Doubling times were extrapolated by noting the time indicated along the axis of the abscissa as each growth curve transected the horizontal lines drawn across (perpendicular to) the ordinate axis.

The doubling times for each compound and its control from all experiments performed were pooled and averaged. The variance and

standard deviation from the mean was computed for the pooled doubling times of sodium and calcium cyclamates and their controls. The Students t statistical test was used to test for a difference between the mean of each experimental compound and its control mean. In addition, analysis of variance and Duncan's new multiple range test were employed to compare all of the means and to test the hypothesis of no differences in doubling times.

Long Term Exposures at Low Concentrations

After observing no obvious effects after short periods of exposure to the cyclamates in relatively high concentrations (1000 ug/ml), experiments were undertaken to determine whether continuous exposure to low concentrations over long periods of time would cause a "permanent" change in growth rate.

For these experiments duplicate cultures were grown on medium supplemented with 1 ug/ml for 97 days and 10 ug/ml sodium cyclamate for up to 134 days.

To determine whether growth rate was altered, two T-25 flasks were seeded with a specified number of treated cells and incubated for 48 hours on normal medium without cyclamate along with two control flasks which were seeded with the same specified number of untreated cells. All cultures were harvested and counted and growth ratios were determined.

The growth rate was determined at three time periods for exposure to 10 ug/ml (97, 124, and 134 days) and one time for exposure to 1 ug/ml (97 days).

Determination of Effects on Later Cell Generations

To ascertain whether effects on growth rate and chromosomes were permanent, temporary or latent in occurrence, the experiments outlined below were conducted.

Two healthy cell cultures in T-75 flasks were exposed to 500 ug/ml sodium cyclamate for 48 hours. At the end of the 48 hour period, the medium containing cyclamate was decanted and replaced by normal growth medium. These cells were then grown as special stock cultures, with regular medium changes and transfers as required, for 90 days. After 90 days cultivation, two T-25 flasks were seeded from one of the special stock flasks and prepared for chromosome studies. Two cultures from normal stock flasks were seeded and prepared as controls. Slide preparation, observations and analyses were done by usual procedures.

To determine whether growth rate was altered, two T-25 flasks were seeded with an equal number of cells from the special stock cultures along with two control flasks seeded with the same number of cells. Cells were harvested and counted and growth ratios were determined as an indicator of growth rates. Growth determinations were made twice using this procedure (after 94 and 106 days).

CHAPTER III

EXPERIMENTAL RESULTS

The results obtained from exposing Chinese hamster cells to various concentrations of the test compounds used in this study are presented in eight sections: effects on chromosome breakage; the distribution of chromosome breaks; effects on chromosome number; effects on growth rate as measured by the metaphase index; growth rates of cells determined by cell counts after 72 hours exposure to compounds; doubling time of cell populations exposed to sodium and calcium cyclamates; effects of long term exposures to low concentrations (1 and 10 ug/ml) of sodium cyclamate on growth rate; effects on later generations of cells. A discussion of each compound is given under each section.

Effects on Chromosome Breakage

Sodium Cyclamate

The results of growing Chinese hamster fibroblasts in the presence of sodium cyclamate for 72 hours at concentrations of 1, 10, 100, 250, 500, and 1000 ug/ml are shown in Figure 1. The numbers of cells with breaks caused by concentrations of 1, 10, 100, 250, and 1000 ug/ml were not significantly different from the number shown by the control. However, the 500 ug/ml concentration caused a highly statistically

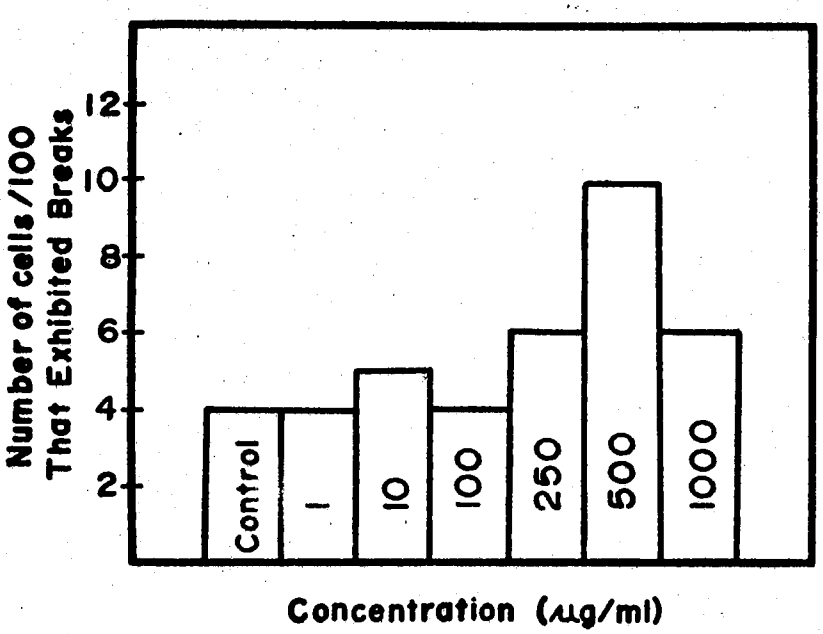


Figure 1. Number of Cells with Chromosome Breaks after Exposure to Various Concentrations of Sodium Cyclamate for 72 Hours

significant increase in the number of cells having broken chromosomes. Table I shows the total number of breaks, number of cells with breaks, probability values and significance levels for the occurrence of cells with breaks. There appeared to be a possible trend of increasing concentration effecting more breaks but this was not consistent.

TABLE I

EFFECTS OF EXPOSURE OF CELLS TO VARIOUS CONCENTRATIONS OF SODIUM CYCLAMATE FOR 72 HOURS

Concentration (ug/ml)	Total Number of Breaks Observed per 100 Cells	Number of Cells that Exhibited Breaks	X^2 No. of Cells with Breaks	P
0	4	4		
1	5	4	0	
10	5	5	0.260	0.75
100	5	4	0	0.90
250	7	6	1.041	0.50
500	14	10	9.3750	0.005
1000	7	5	0.2604	0.75

Although low concentrations of sodium cyclamate did not cause significant breakage of chromosomes after 72 hours of cultivation, it was found that concentrations as low as 10 ug/ml caused a highly statistically significant incidence of cells with breaks after long periods of exposure. After 124 days of exposure to 10 ug/ml of sodium cyclamate, a count of 100 cells revealed that 19 of the 100 cells had at least one chromosome break (Figure 2). The total number of breaks seen in the 100 cells was 27. Seven of the 19 cells (25%) had more than one break. Exposures of 9 and 62 days to 10 ug/ml did not result in significantly increasing breakage over the control for those time periods (Table II). Note however, that in this experiment (Figure 2),

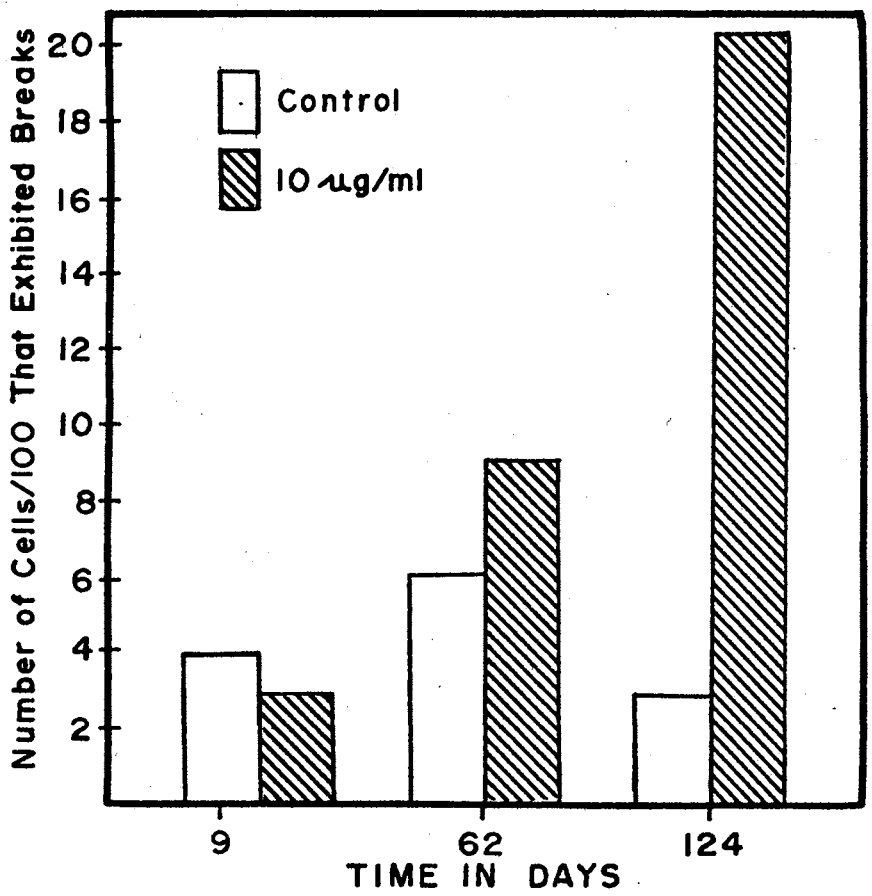


Figure 2. Number of Cells that Exhibited Chromosome Breaks after Prolonged Exposure to 10 ug/ml Sodium Cyclamate

the number of breaks in the control was higher at the 62 day period than at any other sampling time in this study. This could account for the non-significance of the nine breaks found at the 62 day period.

TABLE II

EFFECTS OF EXPOSURE OF CELLS TO 10 ug/ml OF SODIUM CYCLAMATE FOR VARIOUS LENGTHS OF TIME

Length of Exposure (Days)	Total Number of Breaks Observed per 100 Cells	Number of Cells that Exhibited Breaks	X^2 No. of Cells with Breaks	P
9	3	3	0	0.995
Control	4	4		
62	9	9	1.595	0.10
Control	6	6		
124	27	19	87.972	0.005
Control	3	3		

Exposure to 100 ug/ml for 7 days resulted in a highly significant number of breaks, although the number of breaks in the control was higher than average (the 62 day, 10 ug/ml and 100 ug/ml, 7 day samples had the same control). Exposure to 100 ug/ml for 60 days yielded the highest numbers of cells with breaks, total breaks and other aberrations observed in all of the experiments performed (Table III, Figure 3). Figure 3 also shows that the number of cells with breaks increases with time. The number of cells with breaks after 60 days was 20. The total number of breaks found in 100 cells exposed to 100 ug/ml for 60 days was 33. Four cells were observed with figures that appeared to represent fusions of a short metacentric and a subtelocentric chromosome (Figure 4).

TABLE III

EFFECTS OF EXPOSURE OF CELLS TO 100 ug/ml SODIUM CYCLAMATE FOR VARIOUS LENGTHS OF TIME

Length of Exposure (Days)	Total Number of Breaks Observed per 100 Cells	Number of Cells that Exhibited Breaks	X^2 No. of Cells with Breaks	P
3	5	4	0	0.995
Control	4	4		
7	18	14	11.347	0.005
Control	6	6		
60	33	20	47.368	0.005
Control	5	5		

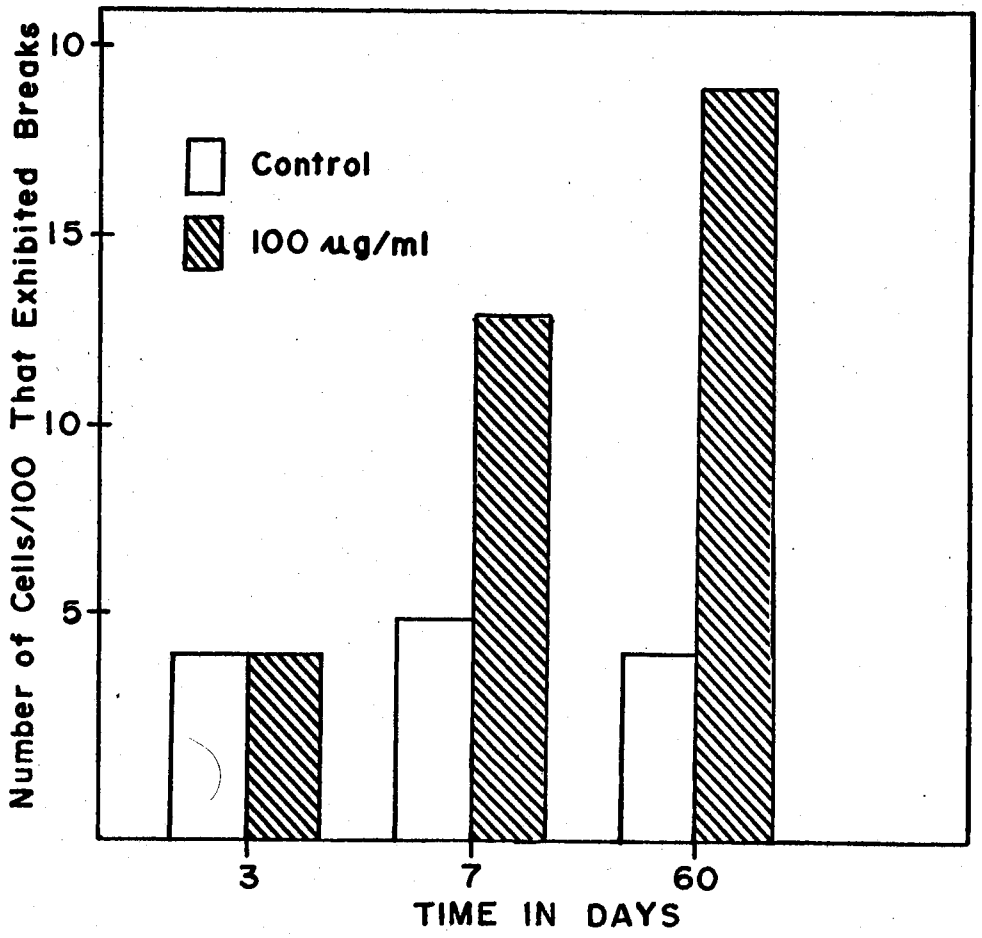
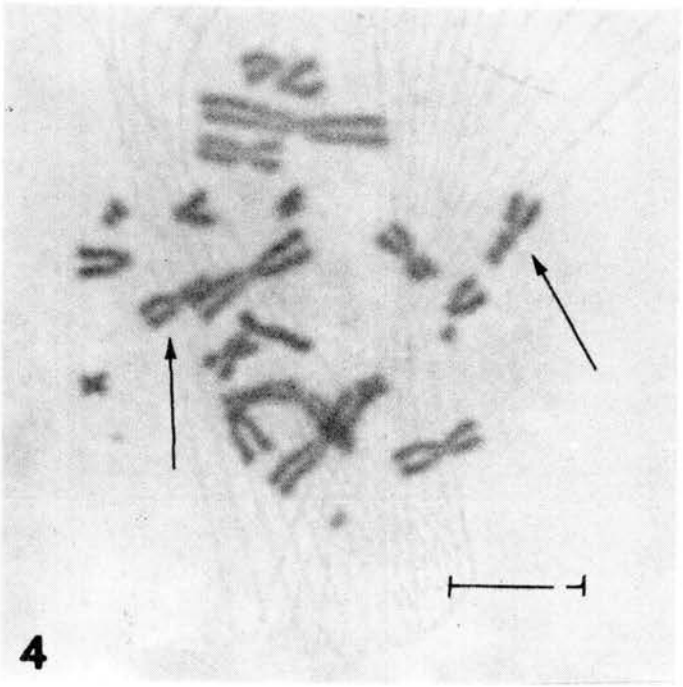


Figure 3. Number of Cells that Exhibited Chromosome Breaks after Prolonged Exposure to 100 ug/ml Sodium Cyclamate

Figure 4. Structural Chromosomal Aberrations that Resemble the Fusion of Acrocentric and Small Metacentric Chromosomes



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Cells exposed to 1 ug/ml of sodium cyclamate for 97 days did not show an increase of cells with breaks over the control. Note from Figure 5 that the number of cells with breaks was less than that of the control. Chromosome breakage was not significant after exposure to 1 ug/ml for 97 days (Table IV).

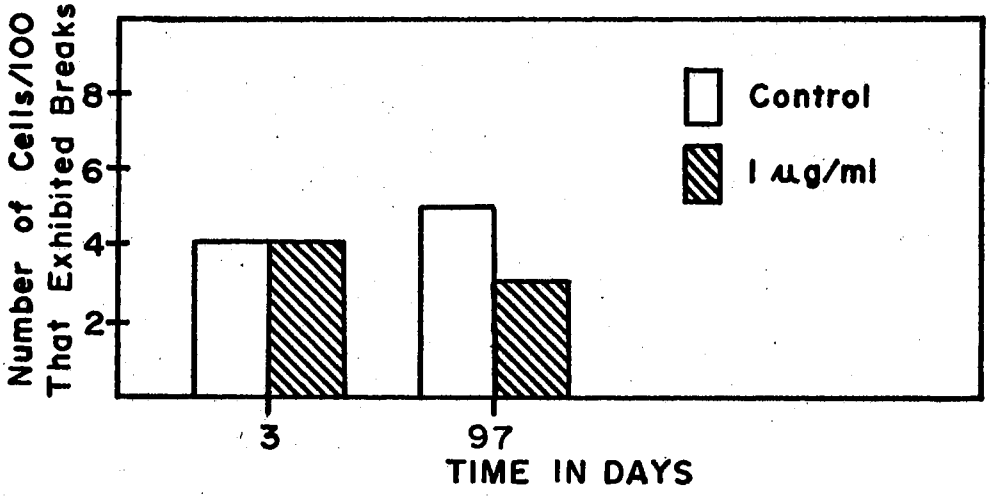


Figure 5. Number of Cells with Chromosome Breaks after Prolonged Exposure to 1 ug/ml Sodium Cyclamate

TABLE IV

EFFECTS OF EXPOSURE OF CELLS TO 1 ug/ml OF SODIUM CYCLAMATE FOR 97 DAYS

Length of Exposure (Days)	Total Number of Breaks Observed per 100 Cells	Number of Cells that Exhibited Breaks	X^2 No. of Cells with Breaks	P
3	5	5	.260	0.75
Control	4	4		
97	3	3	.8421	0.25
Control	5	5		

Results of the long term experiments described above indicate that concentration and length of exposure are factors influencing chromosome breakage produced by sodium cyclamate.

Calcium Cyclamate

Results of observations on 50 metaphases per concentration from cells exposed to 1, 10, 100, 250, 500 and 1000 ug/ml of calcium cyclamate showed that only one concentration, 100 ug/ml, produced a higher percentage of cells with chromosome breaks than the controls. Figure 6 graphically shows that the greatest per cent of cells with breaks was seen in cultures exposed to 100 ug/ml. It appears from the graph that as concentration was increased in excess of 100 ug/ml, the per cent of cells with breaks decreased from this highest point. Table V gives probability values and the total number of breaks found in the 50 cells.

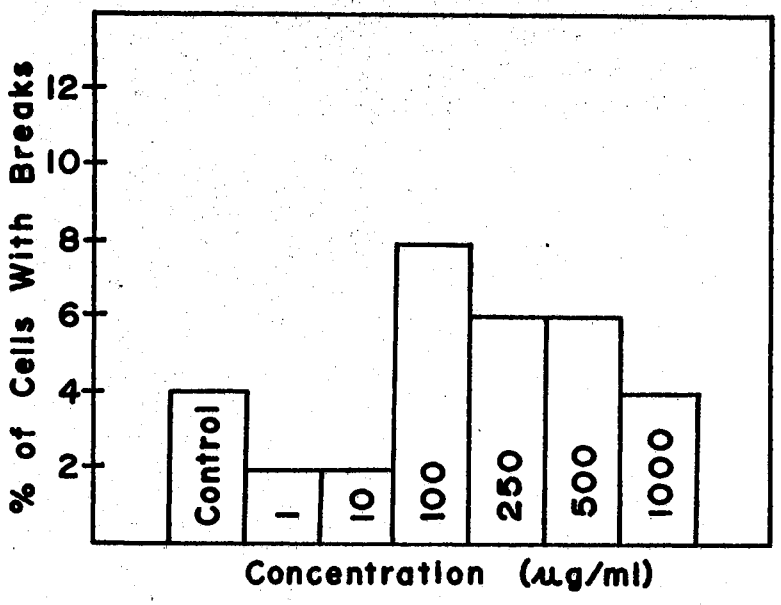


Figure 6. Percent of Cells with Chromosome after Exposure to Various Concentrations of Calcium Cyclamate

TABLE V

EFFECTS OF EXPOSURE OF CELLS TO VARIOUS CONCENTRATIONS OF CALCIUM CYCLAMATE FOR 72 HOURS

Concentration ug/ml	Total Number of Breaks per 50 cells	Number of Cells with Breaks	Percent of Cells with Breaks	X^2 % Cells with Breaks	P
0	7	2	4		
1	2	1	2	1.041	0.25
10	1	1	2	1.041	0.25
100	4	4	8	4.166	0.05
250	3	3	6	1.041	0.25
500	4	3	6	1.041	0.25
1000	2	2	4	0	0.995

Cyclohexylamine

Cyclohexylamine in concentrations of 100 and 250 ul/ml, after 72 hours exposure of the cells, produced a significantly higher percentage of cells with chromosome breaks than were found in the controls. Concentrations less than 250 ul/ml were ineffective in producing a significantly high number of breaks (Table VI, Figure 7. The number of breaks and the percentage of cells with breaks was considerably higher in cells exposed to 100 ul/ml than in those exposed to 250 ul/ml. At the 250 ul/ml concentration only 25 metaphases could be used for analysis because of their scarcity. Under the influence of this concentration (250 ul/ml) the cells appeared shrunken and chromosomes seemed smaller and more compact than in the controls. Fragments of chromosomes and loose or free chromosomes were abundant on these slides.

TABLE VI

EFFECTS OF EXPOSURE OF CELLS TO VARIOUS CONCENTRATIONS OF CYCLOHEXYLAMINE FOR 72 HOURS

Concentration ug/ml	Total Number of Breaks per 50 Cells	Number of Cells with Breaks	Percent of Cells with Breaks	X^2 % Cells with Breaks	P
0	2	2	4		
1	4	3	6	1.041	0.25
10	3	3	6	1.041	0.25
100	9	7	14	26.041	.005
250*	2	2	8	4.166	.05
500	No Metaphases	-	-	-	-
1000	No Cells	-	-	-	-

*Only 25 cells counted because of scarcity of metaphases

The slides of cells exposed to 500 ul/ml cyclohexylamine were void of cells in metaphase. This indicated that cell division was totally inhibited. Examination of flasks after 72 hours exposure to 1000 ul/ml showed no growing cells attached to the surface of the flasks. Therefore, no slides were prepared from this concentration.

Sucrose

The results of exposing cells to concentrations of sucrose as high as 1000 ug/ml are seen in Figure 8. This compound was not effective in causing a significant increase in cells with chromosomal breaks over the control (Table VII).

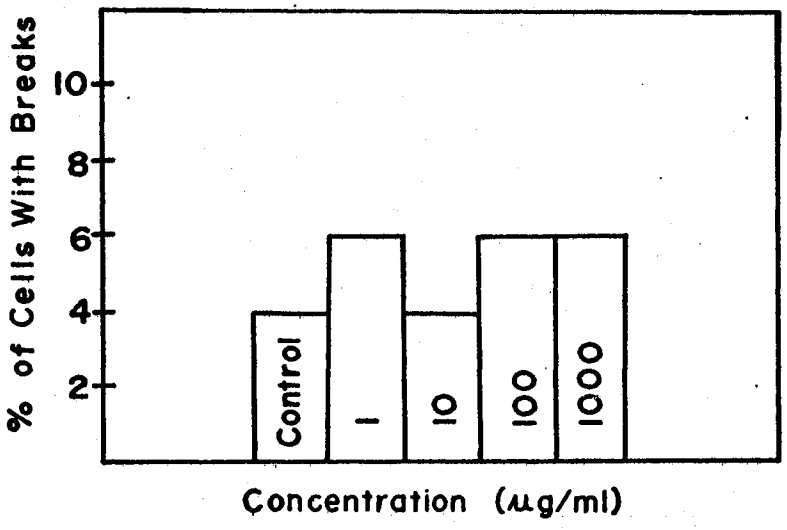


Figure 8. Percent of Cells with Chromosome Breaks after Exposure to Table Sugar (Sucrose) for 72 Hours

TABLE VII

EFFECTS OF EXPOSURE OF CELLS TO VARIOUS CONCENTRATIONS OF TABLE SUGAR (SUCROSE) FOR 72 HOURS

Concentration ug/ml	Total Number of Breaks per 50 Cells	Number of Cells with Breaks	Percent of Cells with Breaks	X^2 % Cells with Breaks	P
0	2	2	4		
1	8	3	6	1.041	0.25
10	4	2	4	0	0.995
100	3	3	6	1.041	0.25
1000	4	3	6	1.041	0.25

Distribution of Chromosome Breaks

To look at the occurrence and distribution of chromosome breaks, notes on observations of those exposures to sodium cyclamate that showed a statistically significant number of breaks over their controls were carefully studied and tabulated. The tabulation included breaks found in the control as well as in experimental cultures.

The number and percent of chromatid and isochromatid breaks found in exposed and control cells with reference to the type of chromosome in which the break occurred are shown in Table VIII. The majority of breaks were of the chromatid type (84%). The majority of breaks occurred in large metacentric chromosomes (80%) and were found in cultures that had been exposed to cyclamate. Seventy-six percent of the number of breaks occurring in large metacentrics (including pairs 1, 2, 4, 5 and the X and Y) were found in a member of pair number one. Most breaks were in the long arm (90%). Table IX gives the number of isochromatid (ISO) and chromatid (CHR) breaks found in each pair of the large metacentric chromosomes. The greatest number of breaks

TABLE VIII

DISTRIBUTION OF CHROMOSOME BREAKS

Type of Chromosome	Chromatid Breaks					Isochromatid Breaks					Total Number of Breaks	Percent of Breaks of the Chromatid Type	Percent of Breaks of the Isochromatid Type
	Number			Percent		Number			Percent				
	Cont	Exptl	Total	Cont	Exptl	Cont	Exptl	Total	Cont	Exptl			
Large Metacentrics	15	59	74	20%	80%	0	14	14	0	100%	88	84%	16%
Acrocentrics	3	19	22	14%	86%	0	6	6	0	100%	28	78%	22%
Small Metacentrics	0	3	3	0%	100%	0	1	1	0	100%	4	75%	25%

120=Total No. of Breaks From All Exposures That Showed Significance

21=Total No. of Breaks of the Isochromatid Type

21=Total No. of Isochromatid Breaks in Exposed Cells

0=Total No. of Isochromatid Breaks in Control Cells

99=Total No. of Breaks of the Chromatid Type

81=Total No. of Chromatid Breaks in Exposed Cells

18=Total No. of Chromatid Breaks in Control Cells

occurred in the number one pair and the smallest number occurred in the sex chromosomes.

TABLE IX

DISTRIBUTION OF BREAKS IN LONG METACENTRIC CHROMOSOMES

	Chromosome									
	1		2		4		5		SEX	
	CHR	ISO	CHR	ISO	CHR	ISO	CHR	ISO	CHR	ISO
Control	12	0	1	0	2	0	0	0	0	0
Experimental	39	6	8	0	8	0	4	1	0	3

The percentages of the total number of breaks found in large metacentrics, acrocentrics and small metacentrics were 73, 23, and 4, respectively. The total number of breaks in control cells (18) was less than that found in exposed cells (102). All breaks found in control cells were of the chromatid type.

The number of chromatid breaks was greater than the number of isochromatid breaks in all chromosomes and groups of chromosomes with one exception. The sex chromosomes (X and Y) had more isochromatid than chromatid breaks (3:0), (Table IX). The percent of isochromatid breaks in acrocentric (29%) was greater than that found in large metacentric chromosomes (20%). No isochromatid breaks were found in small metacentric chromosomes.

Figure 9 shows the normal karotype of Chinese hamster fibroblasts in culture. Figures 10 to 16 show examples of chromosome breaks and other structural abnormalities found in exposed and unexposed cells.

Figure 9. Normal karyotype of fibroblast-like cells of the Chinese hamster *in vitro* (Bar=10 microns)

NORMAL KARYOTYPE

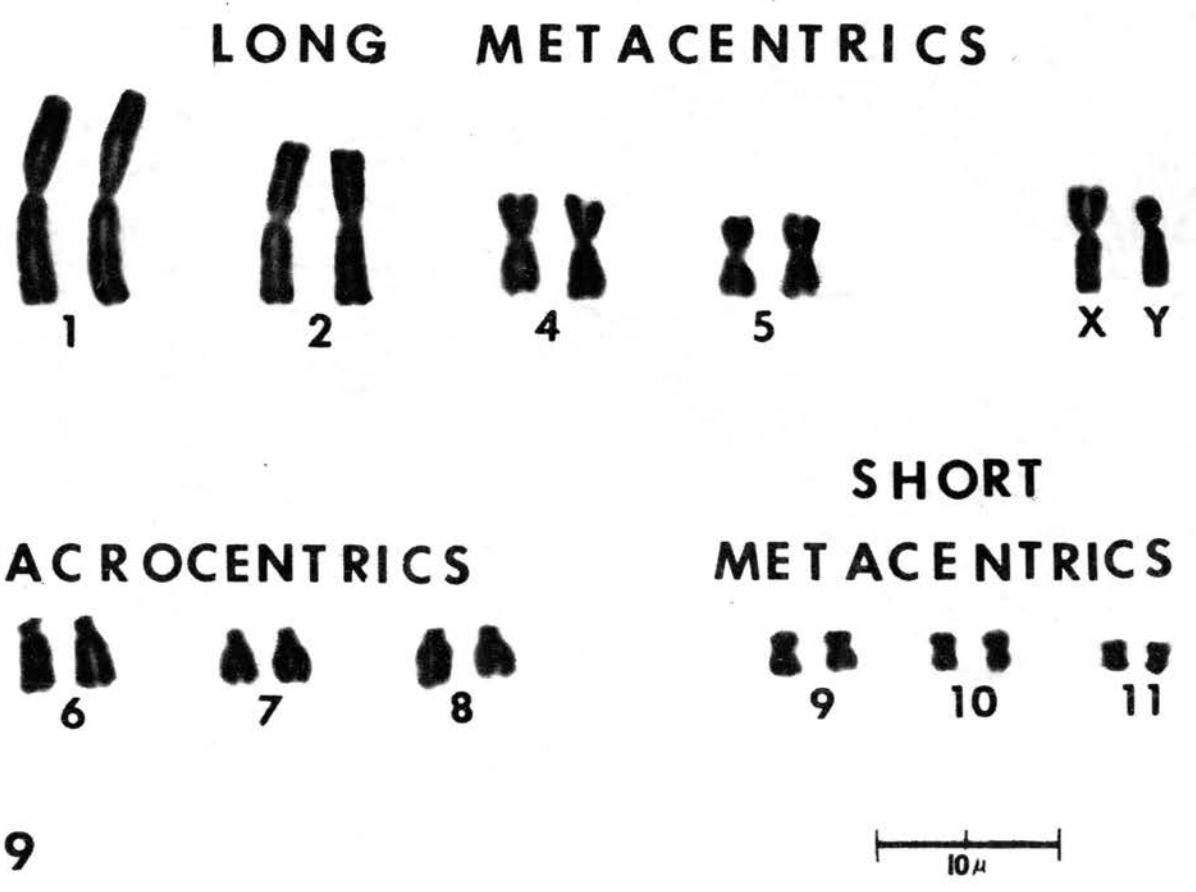
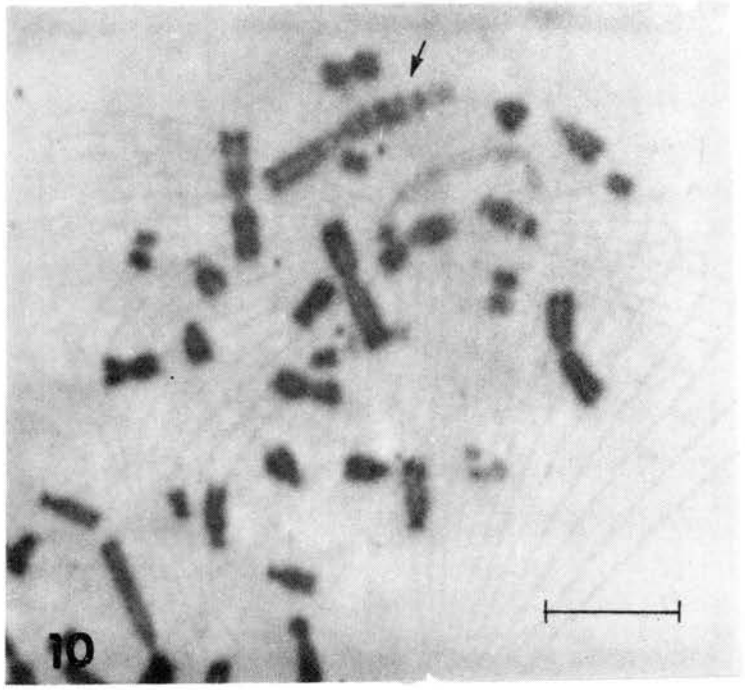


Figure 10. Photomicrograph of a metaphase chromosome spread showing pale staining regions of a chromosome, which were not counted as breaks (Bar=10 microns)

Figure 11. Photomicrograph of a metaphase chromosome spread showing a "gap" in a long metacentric chromosome counted as a break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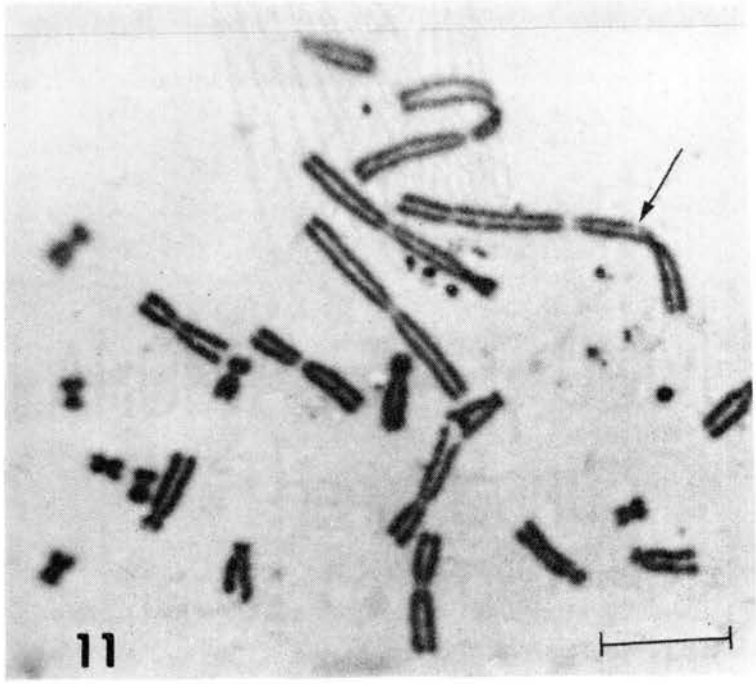
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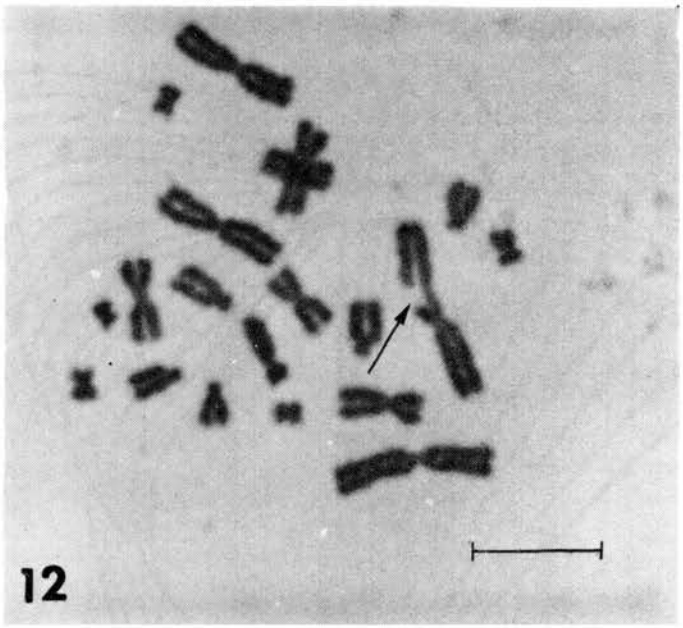
Figure 12. Photomicrograph of a metaphase chromosome spread showing a chromatid break in the long arm of chromosome number one in a cell exposed to 10 ug/ml of sodium cyclamate for 124 days (Bar=10 microns)

Figure 13. Photomicrograph of a metaphase chromosome spread showing an isochromatid break in the long arm of chromosome number two in a cell exposed to 100 ug/ml of sodium cyclamate for seven days (Bar=10 microns)



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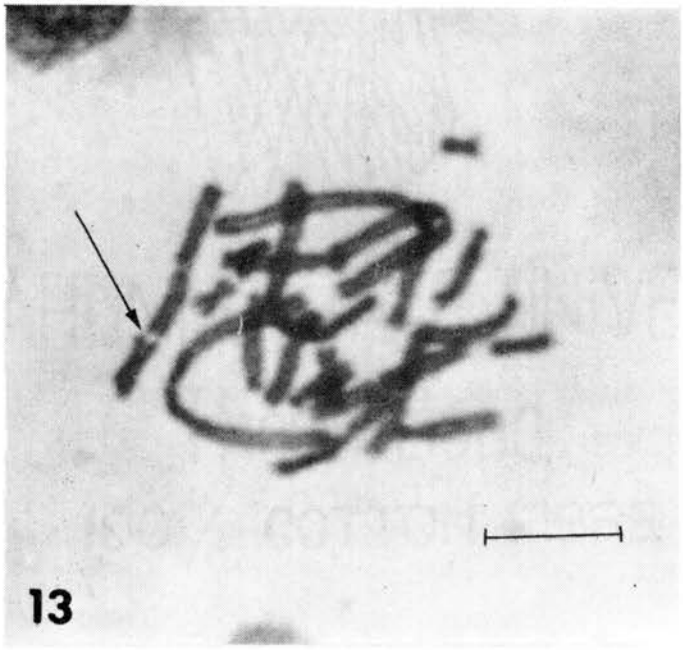

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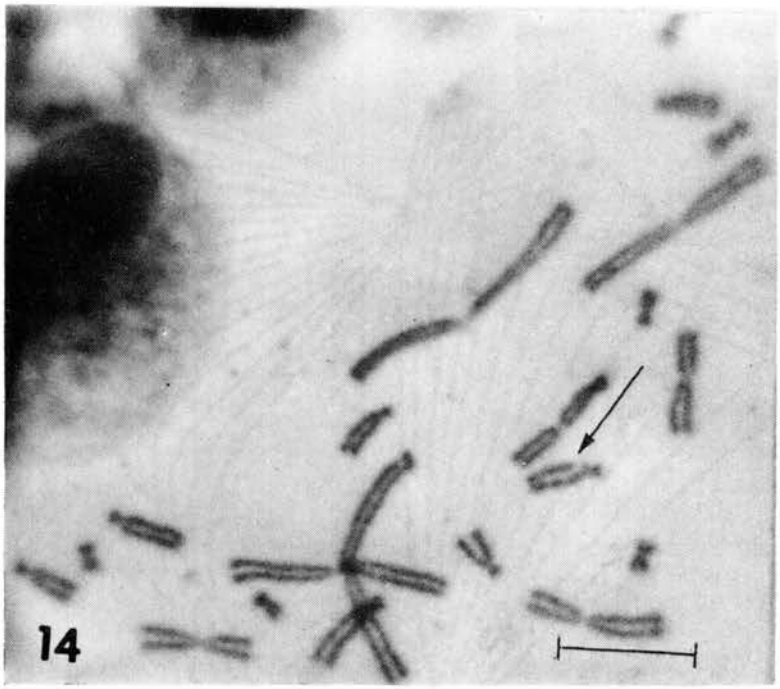
Figure 14. Photomicrograph showing a chromatid break in an acrocentric chromosome from a control cell (Bar=10 microns)

Figure 15. Photomicrograph showing a chromatid exchange in a cell exposed to 100 ug/ml of sodium cyclamate for 60 days (Bar=10 microns)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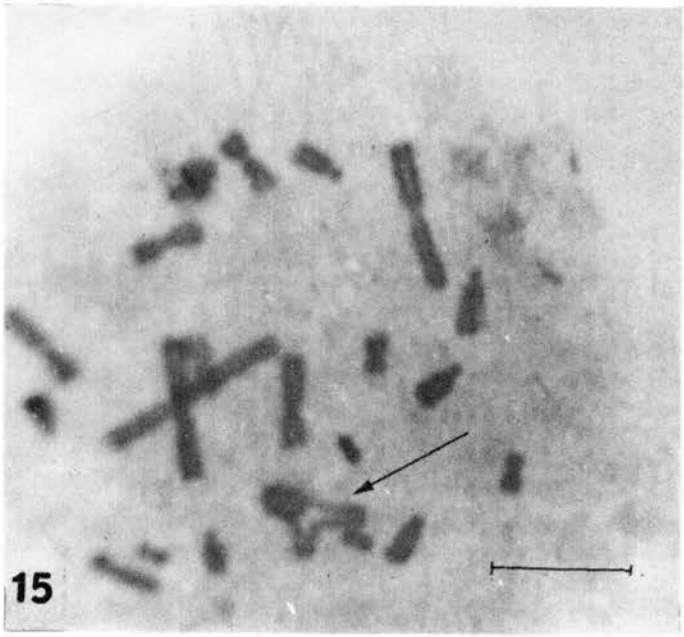
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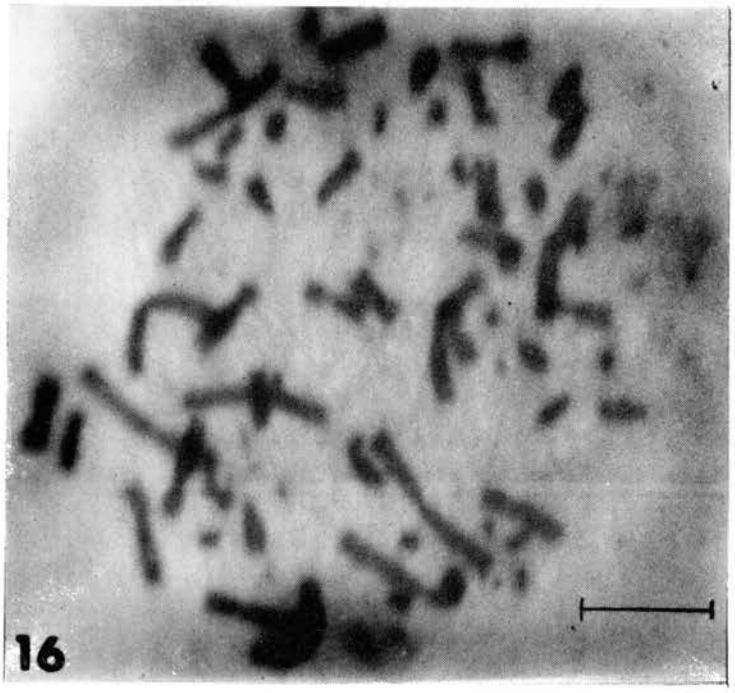
Figure 16. Photomicrograph of a metaphase chromosome spread from a cell exposed to 250 ul/ml of cyclohexylamine showing many fragments of chromosomes (Bar=10 microns)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Effects on Chromosome Number

The number or percent of cells with chromosome numbers that corresponded with the five groups designated for categorization in this study was used as the basis for determining changes in chromosome number as effected by the test compounds. When this grouping varied significantly from that seen in the corresponding control, a significant change in chromosome number was declared. The five groups were given in Materials and Methods.

The chromosome number varied in control cells as well as in experimental cells. The lowest recorded number of chromosomes in controls was 15 and the highest 129. The lowest recorded number in a cell from an experimental group was seven. This cell had been exposed to 250 ul/ml cyclohexylamine for 72 hours. The highest number of chromosomes recorded in experimental cultures was 133, which was seen in a cell exposed to 1 ug/ml sodium cyclamate for 97 days. Figure 17 shows a cell with the normal diploid chromosomal complement (22). Figures 18 - 20 show cells with numerical chromosomal aberrations.

The greatest shift or change in chromosome number from the "approximately diploid" (Group II) was in most cases to Group IV. Further observation revealed that 66% of all the cells in group IV, counted from all experiments, had between 42 and 46 chromosomes. Figures 21, 22, 23, and 24 graphically show that this shift occurred in cultures exposed to all four of the compounds. However, some of the shifts were not statistically significant.

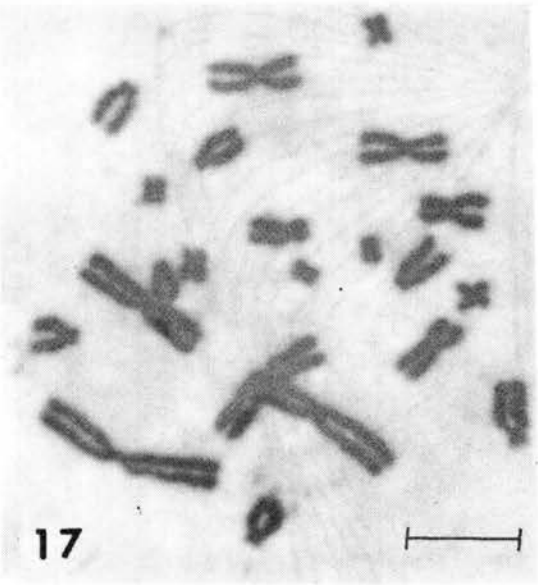
Figure 17. Photomicrograph of a cell with the normal diploid complement of chromosomes (22)

Figure 18. Photomicrograph of a cell with 33 chromosomes

Figure 19. Photomicrograph of a cell with 45 chromosomes

Figure 20. Photomicrograph of a cell with 99 ($\pm$ 2) chromosomes

Bar represents 10 microns



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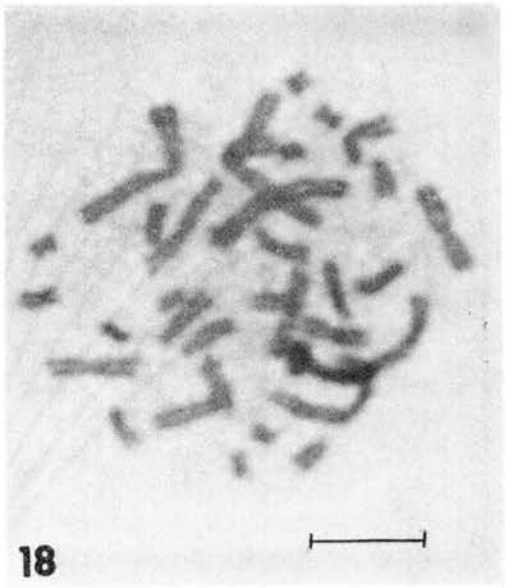

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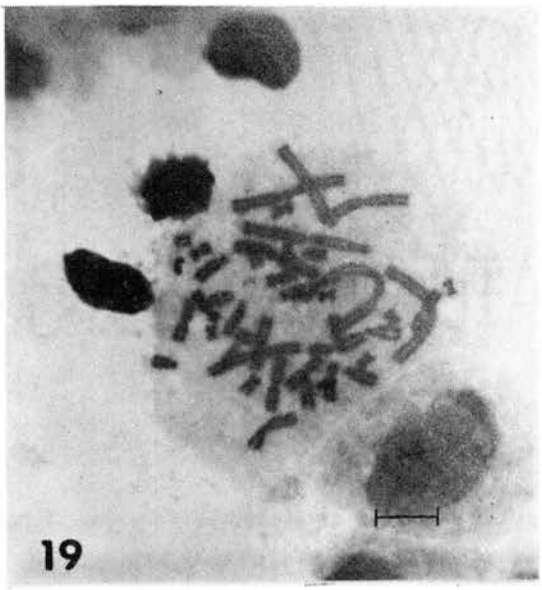

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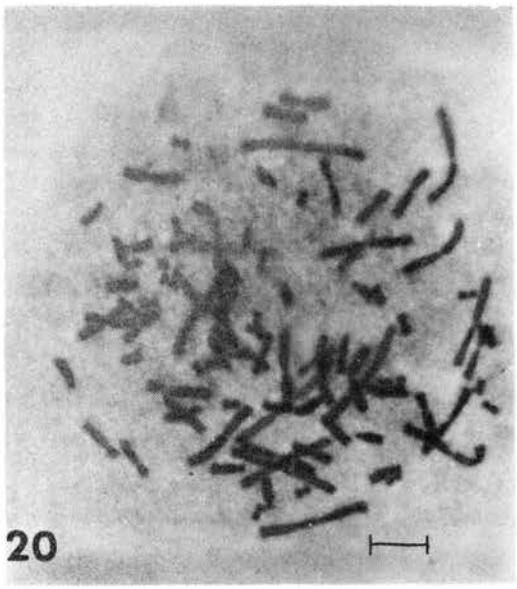

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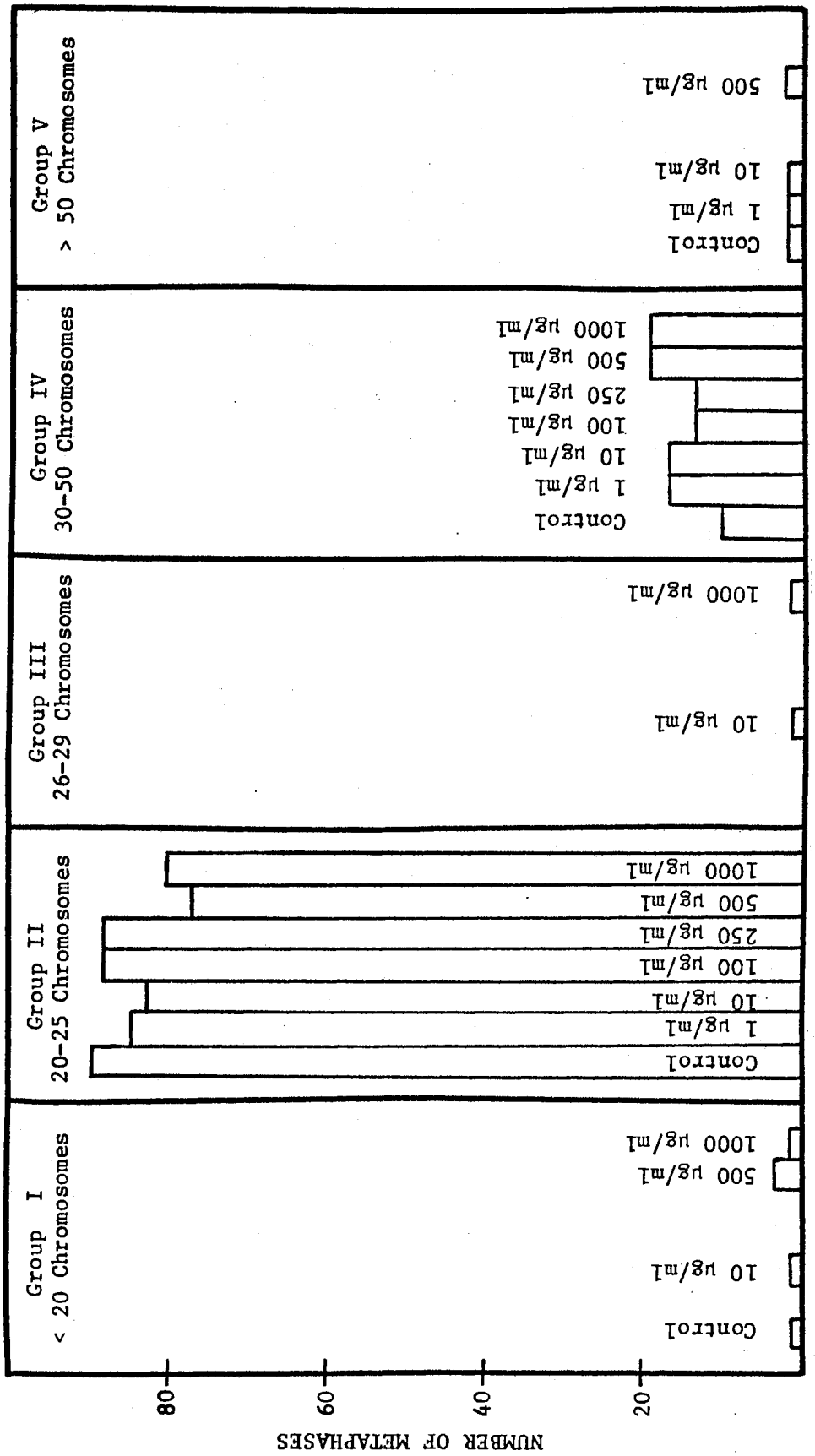

Figure 21. Distribution of 100 Metaphases from Cells Exposed to Various Concentrations of Sodium Cyclamate Based on Chromosome Number

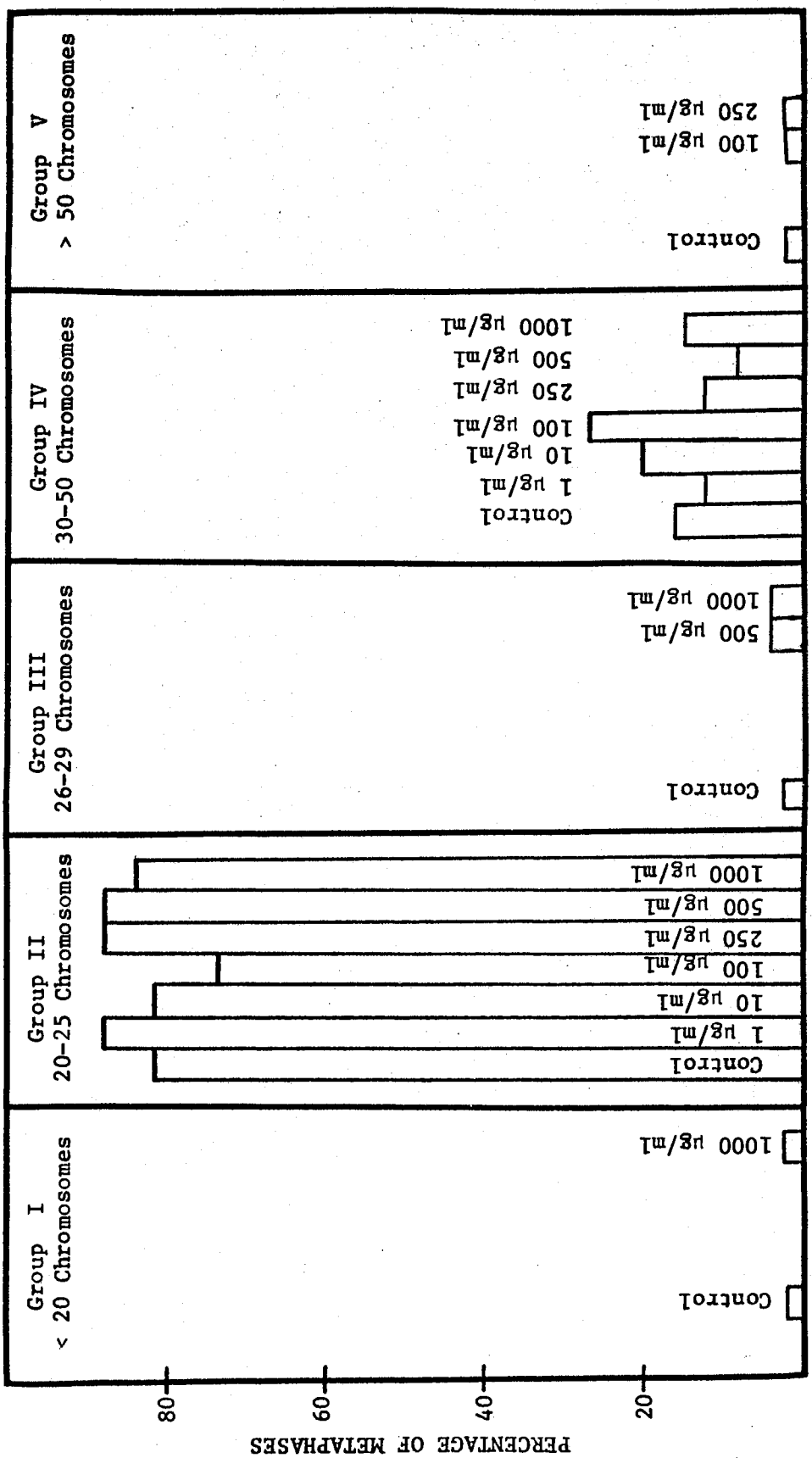

Figure 22. Distribution of 50 Metaphases from Cells Exposed to Various Concentrations of Calcium Cyclamate for 72 Hours Based on Chromosome Number

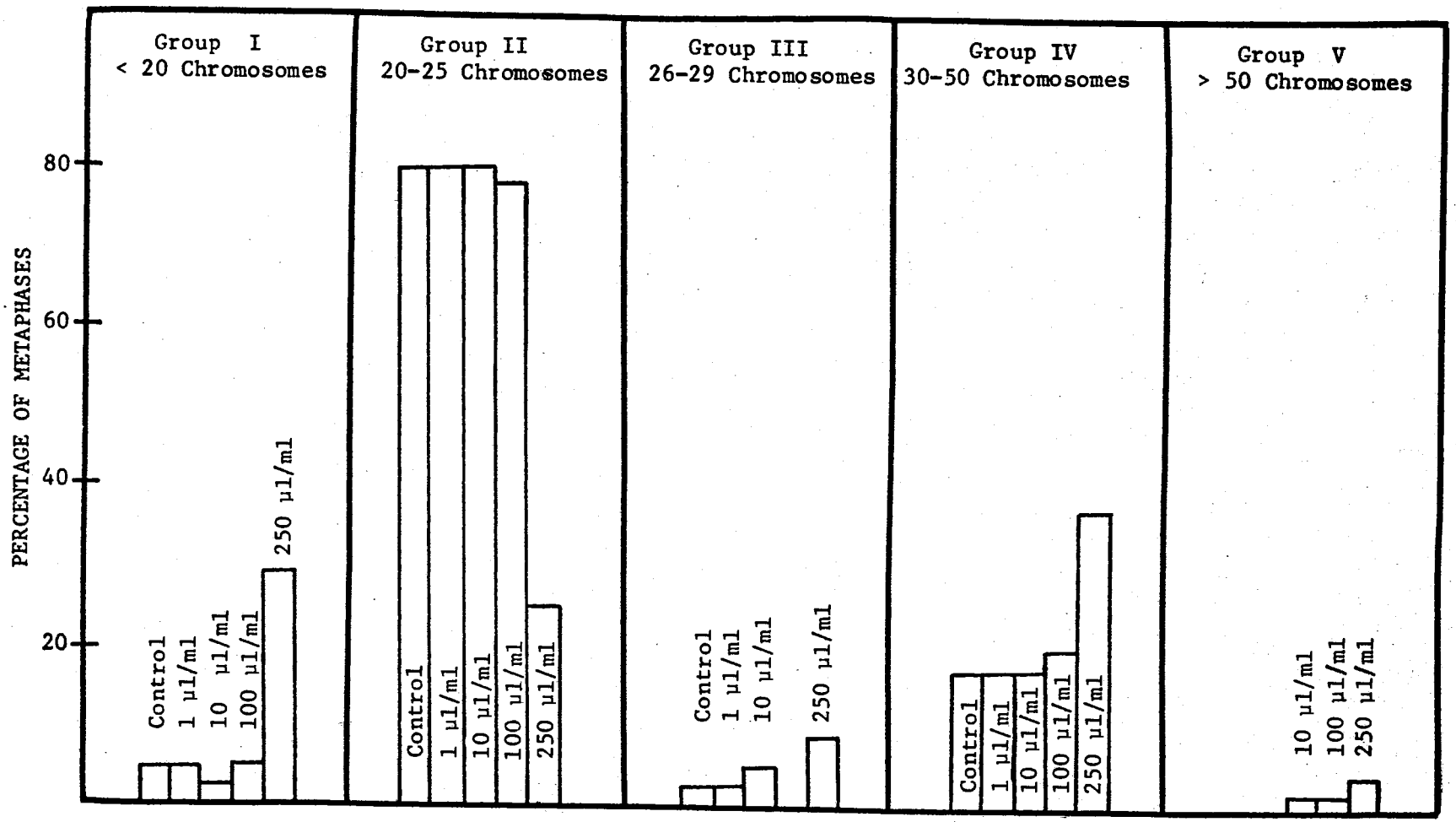

Figure 23. Distribution of 50 Metaphases from Cells Exposed to Various Concentrations of Cyclohexylamine Based on Chromosome Number

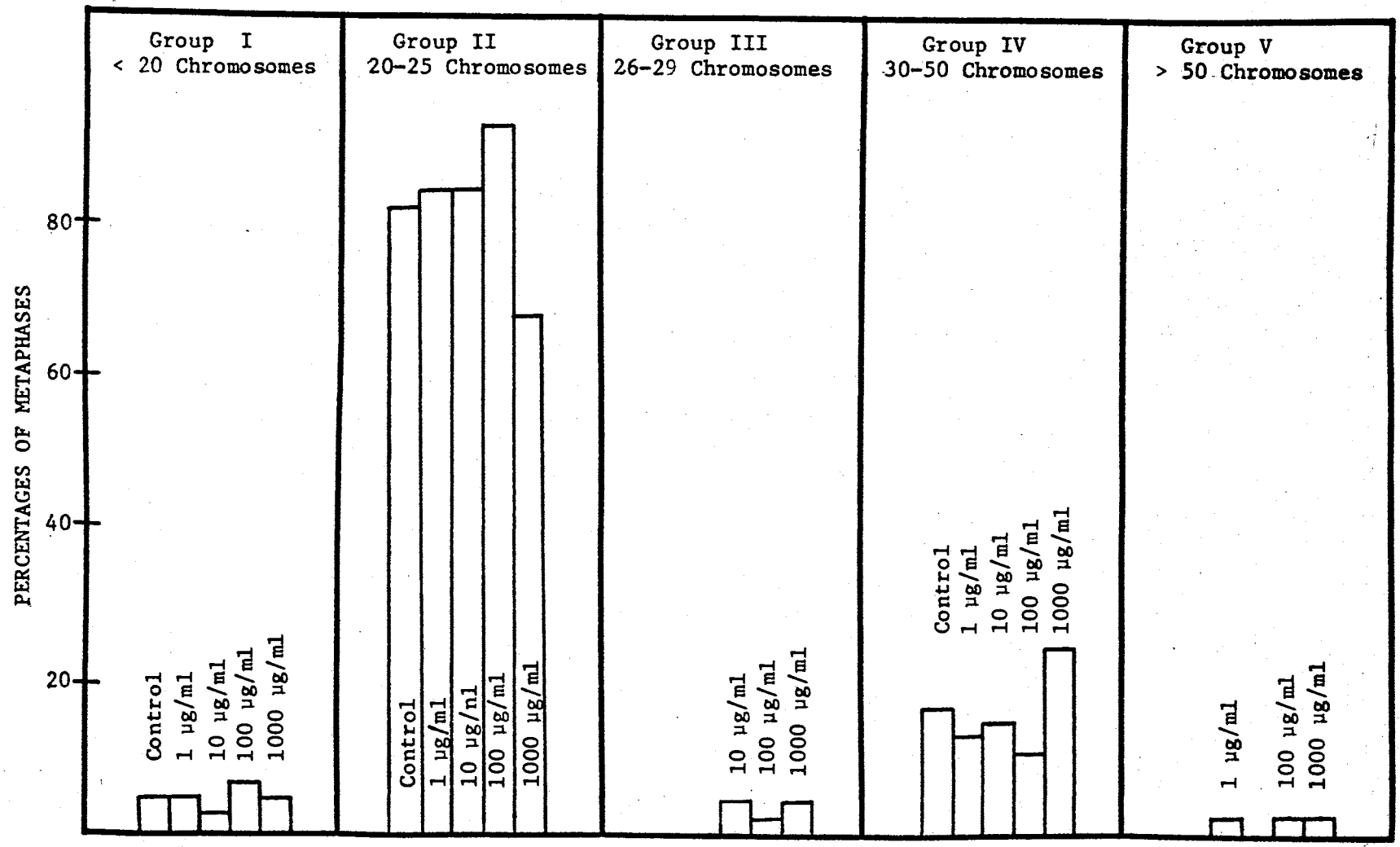

Figure 24. Distribution of 50 Metaphases from Cells Exposed to Various Concentrations of Sucrose Based on Chromosome Number

Sodium Cyclamate

Results showed that cells exposed to the higher concentrations, 500 and 1000 ug/ml, of sodium cyclamate for 72 hours had a higher number of triploid to tetraploid cells and a lower number of diploid cells than found in the control cells not exposed to sodium cyclamate. Chi square values indicated that these changes in number were significant at the 0.05 level. No difference in chromosome number between control cells and cells exposed to concentrations of 250 ug/ml sodium cyclamate and less were noted after 72 hours exposure (Table X).

TABLE X

EFFECTS OF A 72 HOUR EXPOSURE TO SODIUM CYCLAMATE ON CHROMOSOME NUMBER

Concentration ug/ml	Number of Metaphases 20 Group I	20-25 Group II	26-29 Group III	30-50 Group IV	50 Group V	X^2	P
0	1	88	0	10	1	.	.
1	0	83	0	16	1	3.88	0.25
10	1	81	1	16	1	4.15	0.25
100	0	87	0	13	0	1.91	0.75
250	0	87	0	13	0	1.91	0.75
500	3	76	0	19	2	14.73	.01
1000	1	79	1	19	0	19.92	.05

100 cells counted per concentration

Although deviations of chromosome pattern from the control were not observed at low concentrations after 72 hours exposure, highly statistically significant changes at the 0.005 level and greater were observed from cultures that had been exposed to 10 ug/ml for 9, 62, and 124 days and at the 0.01 level from cultures exposed to 100 ug/ml for 7 days. Exposure to 1 ug/ml for 97 days and to 100 ug/ml for 60 days caused no significant changes in chromosome number (Table XI).

TABLE XI

EFFECTS OF LONG TERM EXPOSURES TO SODIUM CYCLAMATE IN VARIOUS CONCENTRATIONS

	Number of Metaphases						
	20 Group I	20-25 Group II	26-29 Group III	30-50 Group IV	50 Group V	X^2	P
3 Day Exposure to 10 ug/ml	1	81	1	16	1	1.9658	0.50
Control	1	88		10	1		
9 Day Exposure to 10 ug/ml	0	73	0	26	1	27.8528	0.005
Control	1	87	1	10	1		
62 Day Exposure to 10 ug/ml	0	82	0	17	1	16.2169	0.01
Control	4	85	0	9	2		
124 Day Exposure to 10 ug/ml	0	78	2	19	1	41.6193	0.005
Control	1	88	0	10	1		
3 Day Exposure to 1 ug/ml	0	83	0	16	1	3.8440	0.25
Control	1	88	0	10	1		
97 Day Exposure to 1 ug/ml	4	84	0	8	0	3.4666	0.25
Control	4	80	0	15	1		
7 Day Exposure to 100 ug/ml	4	72	1	23	0	12.9882	0.025
Control	4	85	0	9	2		
60 Day Exposure to 100 ug/ml	2	80	1	17	0	2.1333	0.75
Control	4	80	0	15	1		

Calcium Cyclamate and Sucrose

Chromosome counts of 50 cells per treatment concentration, after exposure to table sugar (sucrose) in concentrations of 1, 10, 100 and 1000 ug/ml and to calcium cyclamate at concentrations of 1, 10, 100, 250, 500, and 1000 ug/ml, revealed no statistically significant changes in chromosome number as shown in Tables XII and XIII.

TABLE XII

EFFECTS OF A 72 HOUR EXPOSURE TO CALCIUM CYCLAMATE ON CHROMOSOME NUMBER

	Number of Metaphases						
Concentration ug/ml	20 Group I (Percent)	20-25 Group II (Percent)	26-29 Group III (Percent)	20-50 Group IV (Percent)	50 Group V (Percent)	df=4	P
Control	0	80	2	16	2		
1	2	86	0	12	0	2.22	0.50
10	0	80	0	20	0	2.5	0.50
100	0	72	0	26	2	4.34	0.25
250	0	86	0	12	2	1.72	0.75
500	2	86	4	8	0	2.32	0.50
1000	0	82	4	14	0	2.15	0.50

50 cells counted per concentration

TABLE XIII

EFFECTS OF A 72 HOUR EXPOSURE TO TABLE SUGAR (SUCROSE) ON CHROMOSOME NUMBER

Concentration ug/ml	Number of Metaphases 20 Group I (Percent)	20-25 Group II (Percent)	26-29 Group III (Percent)	20-50 Group IV (Percent)	50 Group V (Percent)	df=4	P
Control	4	80	0	16	0		
1	4	82	0	12	2	0.52	0.97
10	2	82	4	14	0	0.15	.99
100	6	90	2	10	2	1.12	0.75
1000	4	66	4	24	2	3.22	0.50

50 cells counted per concentration

Cyclohexylamine

Exposure of cells to cyclohexylamine at a concentration of 250 ul/ml for 72 hours caused a statistically highly significant change in chromosome number whereas concentrations of 100, 10 and 1 ug/ml had no effect on chromosome number. The change involved an increase in 48% of the cells counted for the 250 ug/ml concentration. The number of cells with less than 20 chromosomes was also increased. Less than 25% of the cells exposed to this concentration had the "approximately diploid" number of chromosomes. Table XIV shows that 250 ug/ml of cyclohexylamine caused a greater percent of numerical aberrations than any other concentration or compound studied.

TABLE XIV

EFFECTS OF A 72 HOUR EXPOSURE TO CYCLOHEXYLAMINE ON CHROMOSOME NUMBER IN 50 METAPHASES

Concentration ug/ml	20 Group I (Percent)	20-25 Group II (Percent)	26-29 Group III (Percent)	30-50 Group IV (Percent)	50 Group V (Percent)	df=4	P
Control	4	78	2	16	0		
1	4	78	2	16	0	0	No diff
10	2	78	4	14	2	0.12	.995
100	4	76	0	18	2	0.15	.995
250	28	24	8	36	4	27.69	0.0005
500		No Metaphases					

50 cells counted per concentration

Effects on Growth Rate as Measured by Metaphase Index

Sodium Cyclamate

The number of metaphases per 250 cells counted on each of four slides from each concentration and the control is graphically shown in Figure 25. The mean number of metaphases is also shown, represented by an x. A slight trend of increasing concentrations causing a decrease in growth rate is evident from the graph. Cells exposed to the three highest concentrations (250, 500 and 1000 ug/ml) showed a statistically significant decrease in growth rate as compared to the control. Cells exposed to 1, 10, and 100 ug/ml did not show a significantly different metaphase index from that of the control after the 72 hour exposure. Table XV gives the mean number of

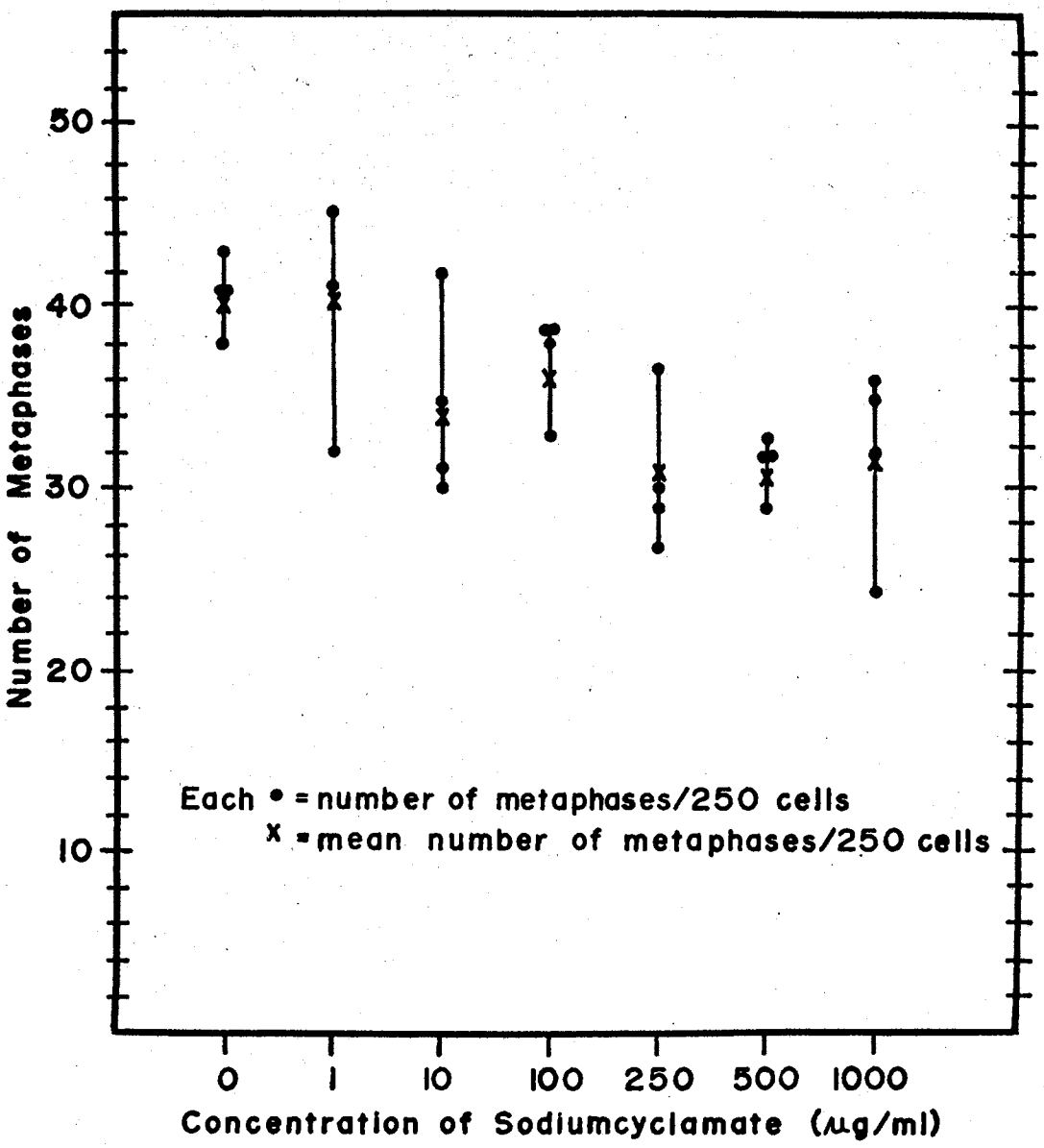

Figure 25. Number of Metaphases per 250 Cell after a 72 Hour Exposure to Various Concentrations of Sodium Cyclamate

metaphases and probability values from the test of the null hypothesis of no difference between the mean of each experimental concentration and the control.

TABLE XV

METAPHASE INDICES FOR CELLS EXPOSED TO VARIOUS CONCENTRATIONS OF SODIUM CYCLAMATE FOR 72 HOURS

Concentration ug/ml	Mean Metaphase Index/250 cells	tw Values	P
0	40.75		
1	40.25	0.055	0.10
10	34.50	0.735	0.05
100	37.25	0.636	0.10
250	30.75	1.333	0.01
500	31.50	2.055	0.01
1000	32.0	1.029	0.05

All cultures exposed to 10 ug/ml of sodium cyclamate for 33 days or longer had a lower metaphase index than control cultures for the same period. These differences were statistically significant at the 0.02 significance level and lower (Table XVI). Cultures exposed to 10 ug/ml for only 9 days showed a greater mean number of metaphases than their controls. Figure 26 shows that the number of metaphases varied considerably from one exposure to another; however, the control was always higher after 33 days exposure. This fluctuation in total numbers did not appear to follow a definite pattern.

TABLE XVI

METAPHASE INDICES FOR CELLS EXPOSED TO 10 ug/ml SODIUM CYCLAMATE FOR A NUMBER OF DAYS

Days Exposure	Mean Metaphase Index/250 cells	tw Values	P
9 Day	20	0.444	0.10
Control	18.5		
33 Day	11.75	2.026	0.01
Control	17.5		
62 Day	21	1.090	0.02
Control	27		
90 Day	1.25	4.333	0.01
Control	7.75		
124 Day	19	2.625	0.01
Control	34.75		

Calcium Cyclamate

Results of exposure of cells to calcium cyclamate showed that the metaphase index was highest in the control cultures and lowest in the cultures exposed to 1000 ug/ml. The number of metaphases was inversely proportional to the concentration of the compound. As concentration was increased from 1 to 1000 ug/ml the number of metaphases decreased (Figure 27). However, the mean indices for the 10 and 100 ug/ml concentrations were the same. A statistically significant difference, at the 0.05 level of significance (t_w, test), was found between the control and all concentrations except 1 ug/ml (Table XVII)

Cyclohexylamine

Exposure of cells to all concentrations of cyclohexylamine for 72 hours caused a statistically significant decrease in growth rate

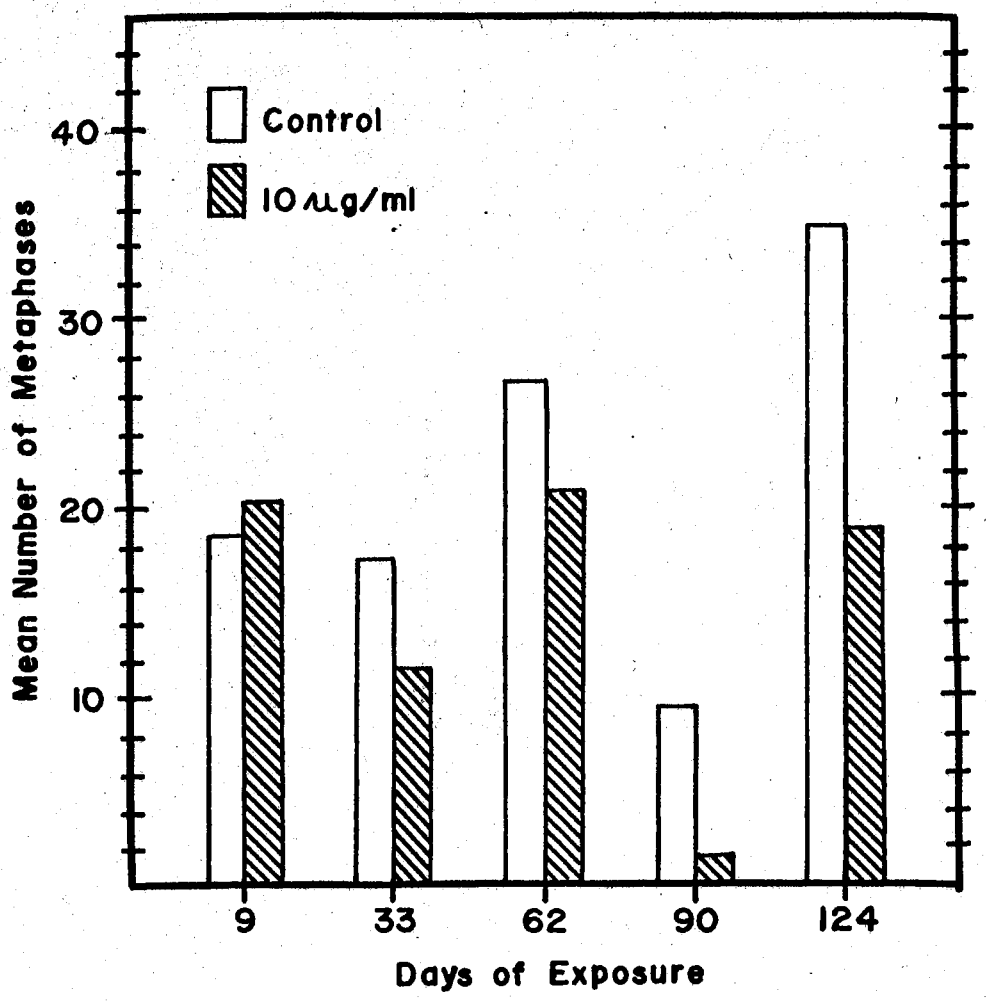

Figure 26. Mean Number of Metaphases per 250 Cells after Exposure to 10 ug/ml Sodium Cyclamate for Several Days

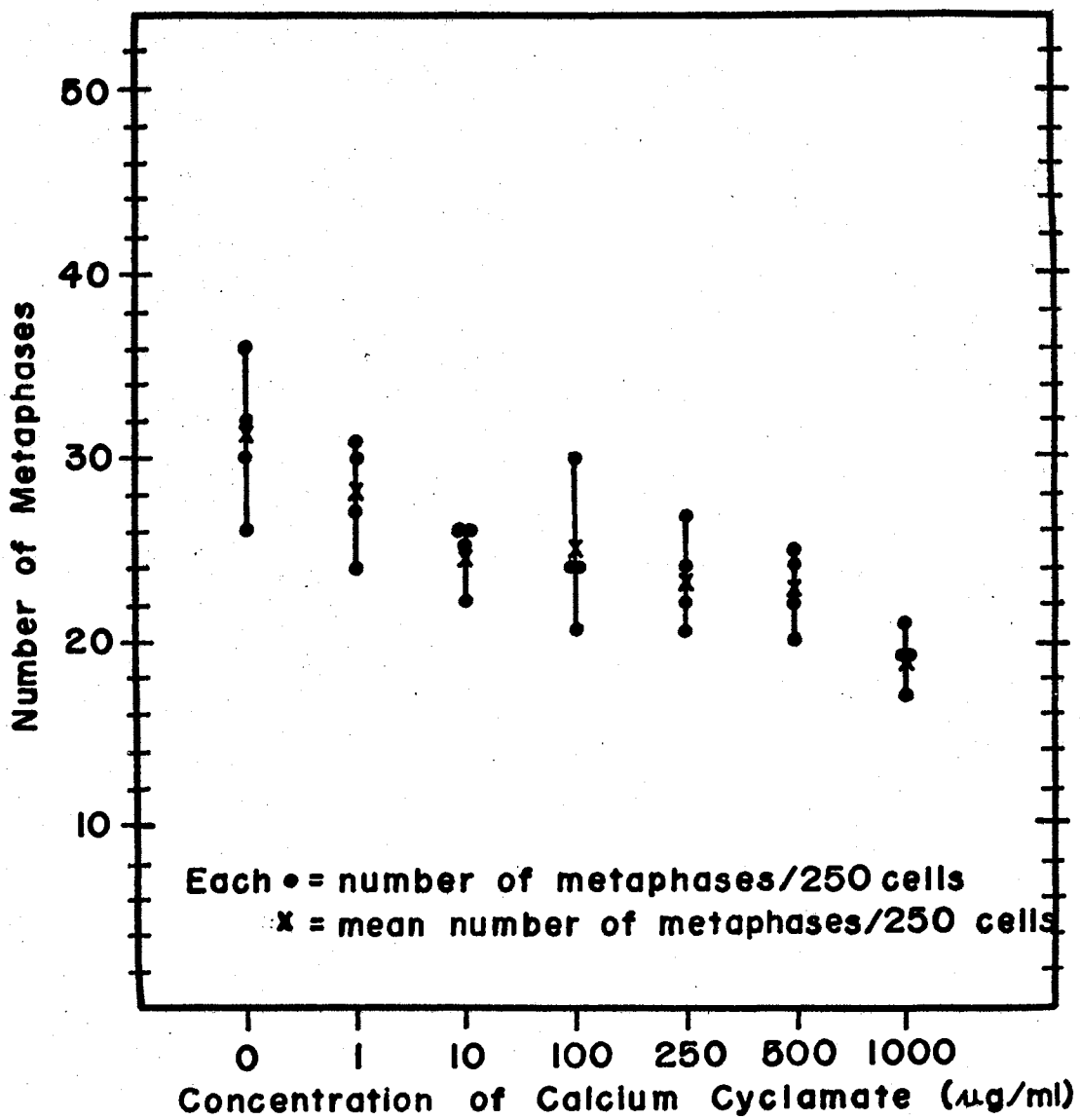

Figure 27. Number of Metaphases per 250 Cells after a 72 Hour Exposure to Various Concentrations of Calcium Cyclamate

as compared to the control rate (Table XVIII). The number of metaphases observed was inversely proportional to concentration. The highest index was found in controls and the lowest in cultures exposed to 250 ug/ml cyclohexylamine (Figure 28). Cell division was completely inhibited in cultures exposed to 500 ug/ml. Cell density was very low on these slides. No metaphases were observed. Examination of cultures exposed to 1000 ug/ml cyclohexylamine revealed that no cells were attached to the flasks. This concentration was considered lethal to Chinese hamster cells.

TABLE XVII

METAPHASE INDICES FOR CELLS EXPOSED TO VARIOUS CONCENTRATIONS OF CALCIUM CYCLAMATE FOR 72 HOURS

Concentration ug/ml	Mean Metaphase Index/250 cells	tw Values	P
0	31.50		
1	28.00	0.352	0.10
10	24.75	0.892	0.05
100	24.75	0.781	0.05
250	23.50	1.142	0.01
500	22.75	1.100	0.02
1000	19.0	1.785	0.01

TABLE XVIII

METAPHASE INDICES FOR CELLS EXPOSED TO VARIOUS CONCENTRATIONS OF CYCLOHEXYLAMINE FOR 72 HOURS

Concentration ul/ml	Mean Metaphase Index/250 cells	tw Values	P
0	29.0		
1	19.5	0.826	0.05
10	13.5	1.555	0.01
100	12.5	1.650	0.01
250	4.0	2.500	0.01
500	0	0	-
1000	No cells	0	-

Sucrose

The metaphase index of cultures exposed to 1000 ug/ml of sucrose for 72 hours was not significantly different from that of control cells. Figure 29 shows the number of metaphases per 250 cells from the four slides counted. Cultures exposed to 1, 10, and 100 ug/ml showed a statistically significant faster growth rate than control cells. The highest mean index was observed from cultures exposed to 100 ug/ml (Table XIX).

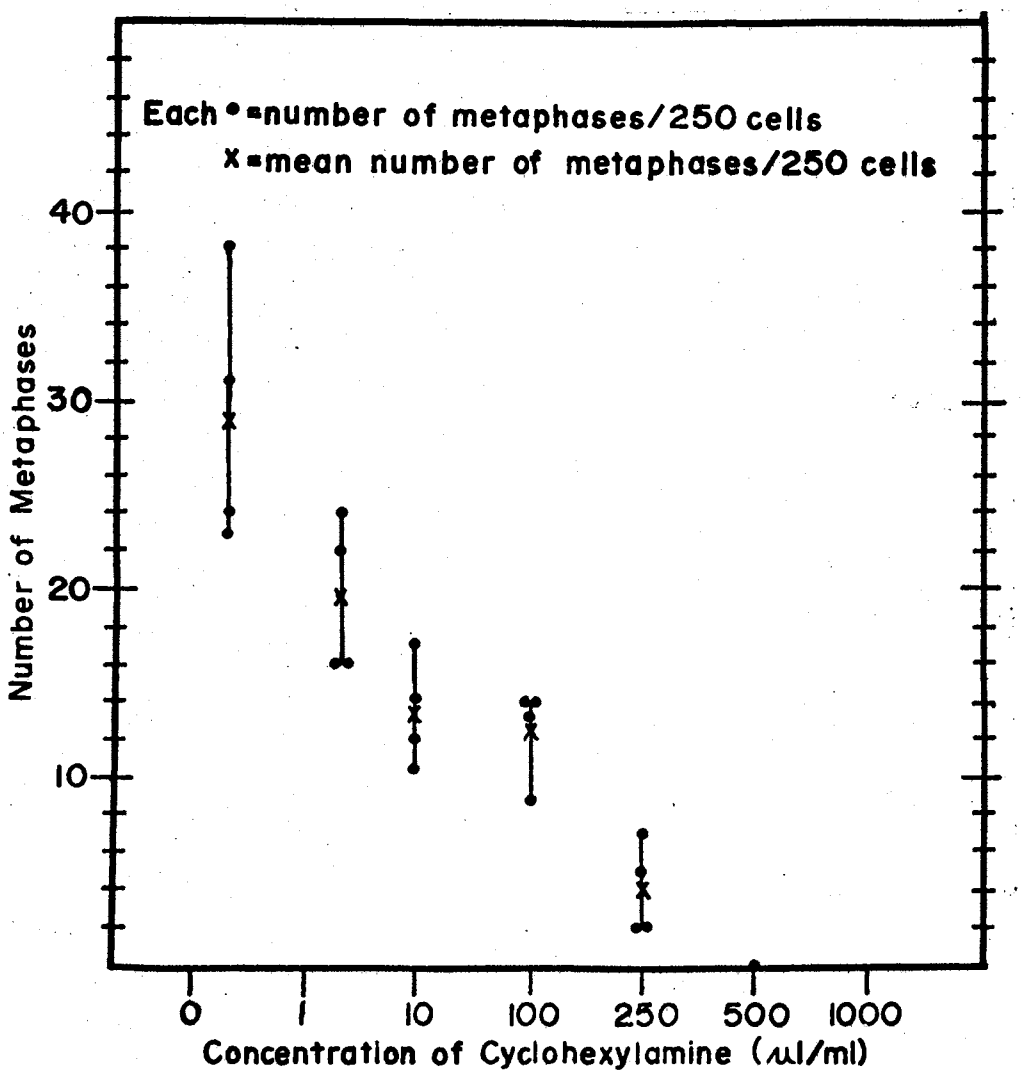

Figure 28. Number of Metaphases per 250 Cells after a 72 Hour Exposure to Various Concentrations of Cyclohexylamine

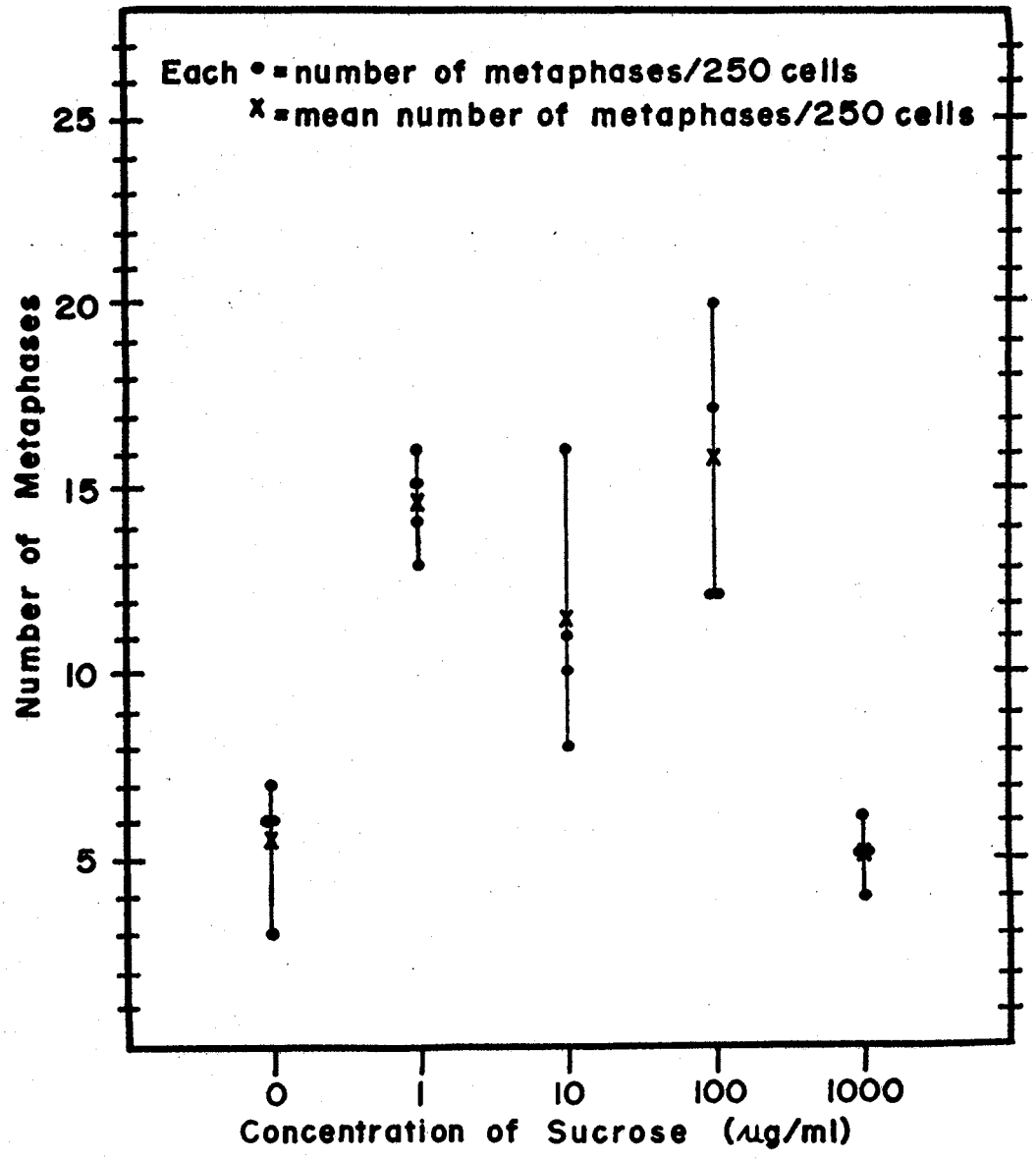

Figure 29. Number of Metaphases per 250 Cells after a 72 Hour Exposure to Various Concentrations of Sucrose

TABLE XIX

METAPHASE INDICES FOR CELLS EXPOSED TO VARIOUS CONCENTRATIONS OF TABLE SUGAR (SUCROSE) FOR 72 HOURS

Concentration ug/ml	Mean Metaphase Index/250 cells	tw Values	P
0	5.5		
1	14.5	-2.571	0.01
10	11.25	-0.958	0.02
100	15.75	-1.863	0.01
1000	5.0	0.166	0.10

Growth Rates of Cells Determined by Cell Counts after 72 Hours Exposure to Test Compounds

Sodium Cyclamate

The growth ratios (rates compared to control rate) of cells exposed to sodium cyclamate for 72 hours are shown in Figure 30. Growth rate determinations on cells exposed to concentrations of 1, 5000, 10^4 and 10^5 ug/ml were done only once. These are represented on the graph by one point each. The latter three extremely high concentrations (5000, 10^4 and 10^5 ug/ml) were studied only to find out whether any effects at all could be obtained with this compound. These concentrations were not within the concentration range that the author felt was relevant for further study.

Growth ratios for 0 (control), 10, 100 and 1000 ug/ml were calculated five times from five different experiments. These ratios

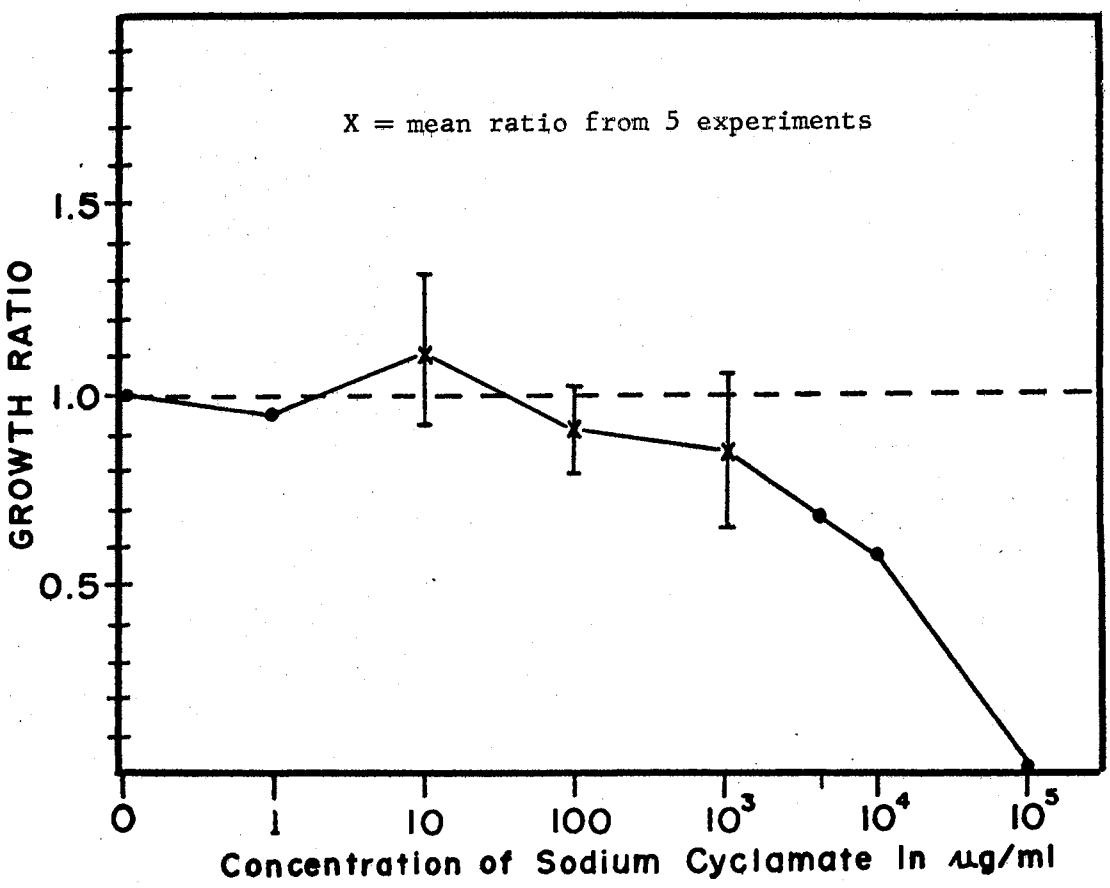

Figure 30. Growth Ratios after Exposure to Sodium Cyclamate for 72 Hours

are found in Table XX. The mean ratios of these five determinations are plotted in Figure 30. Standard error of the mean for each of these three concentrations from the five experiments is also shown in this figure.

TABLE XX

GROWTH RATIOS AFTER EXPOSURE TO THREE CONCENTRATIONS OF SODIUM CYCLAMATE FOR 72 HOURS

Experiment Number	Growth Ratio			
	0 ug/ml	10 ug/ml	100 ug/ml	1000 ug/ml
1	1.000	1.153	1.278	1.606
2	1.000	1.778	0.916	0.835
3	1.000	1.568	0.630	0.516
4	1.000	0.894	0.790	0.726
5	1.000	1.182	0.901	0.623
X	5.000	5.575	4.515	4.307
$\bar{X}$	1.00	1.115	0.903	0.861
S.E.	0	0.199	0.104	0.193
LSD .05	NS	NS	NS	NS

NS=Not significant at 0.05 level when compared with control

On the average, cells exposed to 10 ug/ml grew 11% faster than the control cells. Cells exposed to 1, 100, and 1000 ug/ml grew at rates which were, on the average, 7, 10 and 14% slower than control cells, respectively. However, an F test at the 0.05 significance level using analysis of variance showed that there were no statistically significant differences among the growth ratios of cells exposed to 0, 10, 100 and 1000 ug/ml (Table XXI). A least significance difference test (LSD) showed no statistically significant difference between the mean ratio of the control and the mean ratio of each of these concentrations (Table XX).

TABLE XXI

ANALYSIS OF VARIANCE FOR DIFFERENCES BETWEEN GROWTH RATIOS OF CELLS EXPOSED TO 0, 10, 100 AND 1000 ug/ml SODIUM CYCLAMATE

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Sum of Squares	F
Total	19	1.96	0.103	
Between Concentrations	3	0.19	0.063	0.572
Within Concentrations	16	1.77	0.111	

F.05; 3, 16 df=3.24

Exposure to 5000 and 10^4 ug/ml reduced the growth rate of cells by 33 and 43% respectively. Exposure to 10^5 ug/ml completely inhibited growth and proliferation of cells.

No outstanding differences in attachment, shape of cells, pH, or degree of confluency between control cultures and cultures supplemented with up to 1000 ug/ml of sodium cyclamate were observed. At concentrations of 5000 and 10^4 ug/ml, cell morphology, general appearance and attachment mode were not different from the controls. The pH of the medium on these cultures was not as acidic as that of the controls as indicated by the light orange color of the medium. Control culture medium was light yellow after the 72 hour exposure. The monolayers of cells attached to the surface of flasks were less dense than control layers. In cultures exposed to 10^5 ug/ml, very few cells were attached to the surface of the flasks. The majority of the attached cells were rounded and very granular and vacuolated.

Very few were stellate and glossy in appearance, as were controls. No monolayer sheet was visible. The attached cells appeared as singles.

Calcium Cyclamate

The growth rates of cells exposed to 1, 10, 500, and 1000 ug/ml for 72 hours were not appreciably different from those of the control cells. Results indicated that growth rates were within 14% of that of the control for these concentrations. The growth ratio of cells exposed to 100 ug/ml for 72 hours, however, indicated that this concentration caused an increase in growth rate of 52%. Figure 31 shows the growth ratios for this experiment. Also shown in this figure are growth ratios of an adjunct experiment where cells were exposed to calcium cyclamate at these same concentrations for 24 hours. Results of this experiment indicated that the 100 ug/ml concentration caused a decrease in growth rate of 36%. The results with the other concentrations (1, 10, 500, 1000 ug/ml) studied in the 24 hour experiment agreed with those of the 72 hour study. Hence, the disproportionate 52% increase in the 72 hour exposure may not have been real.

The pH as indicated by the color of the phenol red indicator in the medium showed no apparent differences between the control and experimental cultures. Attachment, degree of confluency and general appearance of the monolayer sheet of cells were similar for the control and experimental cultures.

Cyclohexylamine

Cultures exposed to cyclohexylamine at a concentration of 10 ul/ml showed a growth ratio of 1.085. This indicated that these

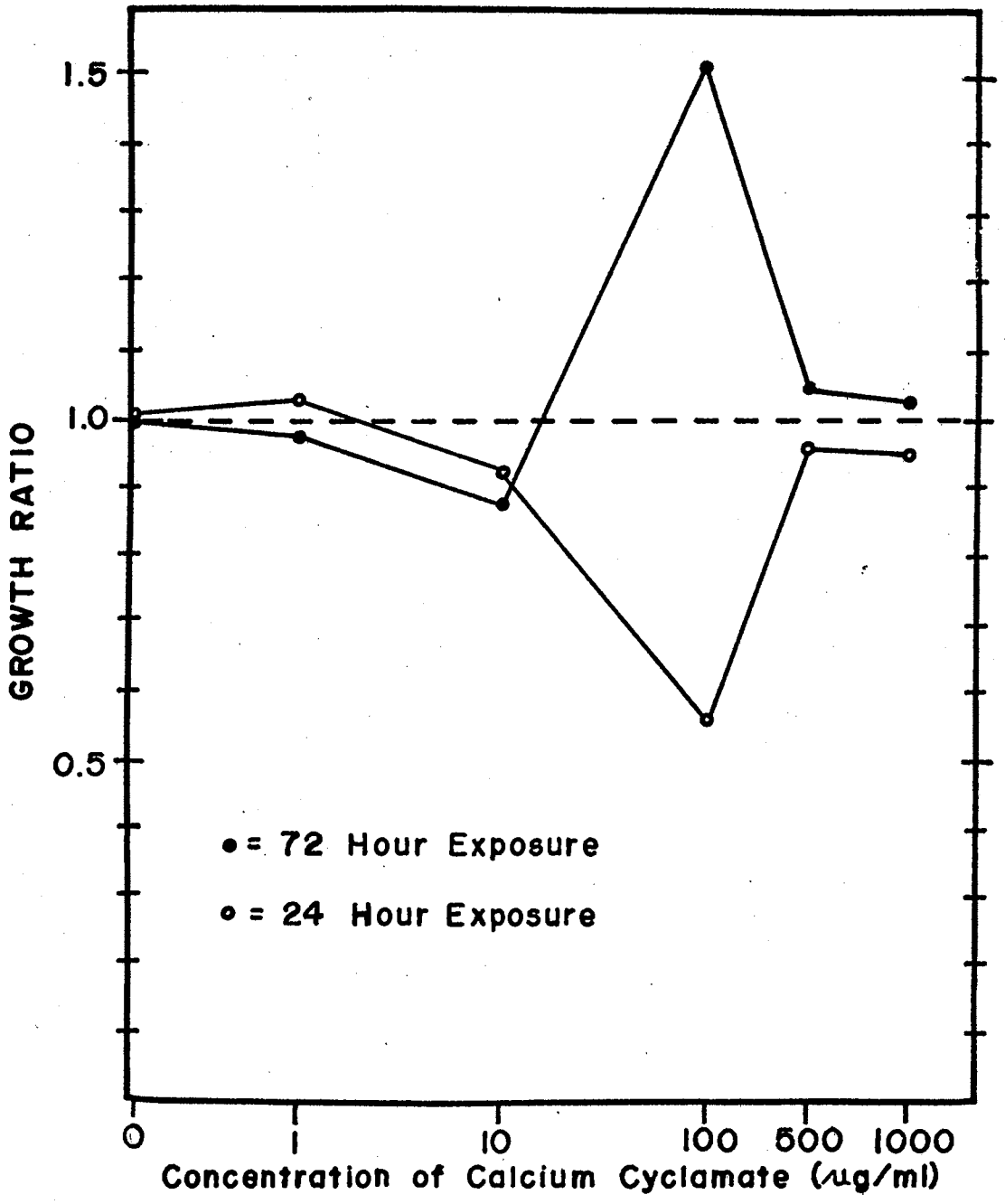

Figure 31. Growth Ratios of Cells after 72 and 24 Hour Exposures to Various Concentrations of Calcium Cyclamate

cultures were growing at a rate which was 8.5% faster than the control cells. No differences from the control in the degree of confluency, pH or attachment were noted.

Exposure to 100 and 1000 ug/ml caused significant decreases in growth rates of approximately 56 and 99.6%, respectively, as compared to the control. Growth ratios are graphically shown in Figure 32. Cultures exposed to 100 ug/ml showed good attachment and no apparent changes in appearance or pH from that of the control cultures. However, the cell layer was noticeably thinner and less confluent than the thick confluent layer of control cells. Cultures under the influence of 1000 ug/ml showed marked differences in pH, attachment, degree of confluency and cell morphology from control cultures. The cells were rounded, very granular, vacuolar and sparse as compared to the abundant stellate shaped glistening cells in control cultures. The color of the medium was a deep pink to purple which indicated that metabolism was affected. The medium on control cultures was yellow which indicated that they were actively metabolizing and releasing carbon dioxide which made the medium acidic (yellow). Only a very few cells were attached to the surfaces of flasks exposed to 1000 ug/ml. Most cells were floating and dead. Many cell fragments were seen microscopically. This concentration was considered to be lethal.

Sucrose

Growth ratios of cells exposed to 1, 10, and 1000 ug/ml of sucrose for 72 hours was less than 1.0 whereas the growth ratio of cells exposed to 100 ug/ml for this same period was greater than 1.0.

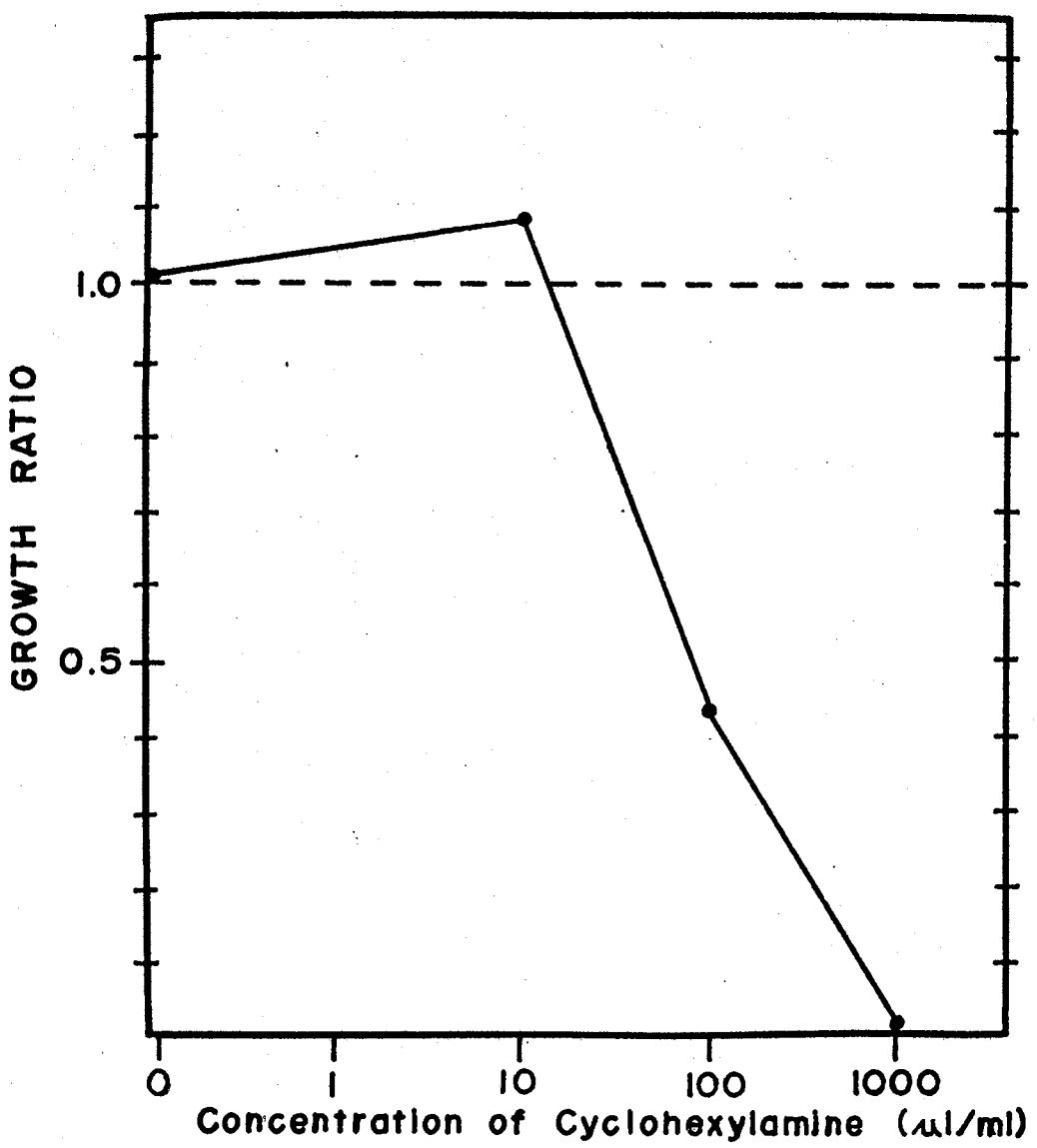

Figure 32. Growth Ratios of Cells after 72 Hours Exposure to Various Concentrations of Cyclohexylamine

Figure 33 graphically shows these ratios which indicate that cells exposed to the former three concentration (1, 10, and 1000 ug/ml) were growing at rates which were approximately 11, 2 and 23%, respectively, slower than control cell growth rate. The growth ratio of 1.23 for cultures exposed to 100 ug/ml indicates that these cells were growing 23 percent faster than the control cells.

Macroscopic and microscopic observation of cultures before harvest revealed that pH, morphology and attachment were approximately the same for all cultures (control and experimental). However, some cells in cultures treated with 1000 ug/ml appeared rounded and were not as stellate and glossy in appearance as control cells. These cultures were also less dense, as observed from the monolayer.

Doubling Time of Cells Exposed to Sodium and Calcium Cyclamates

The doubling times of cells treated with sodium and calcium cyclamates in concentrations of 1000 ug/ml were calculated from a plot of number of cells versus time over a 48 hour period as seen from Figures 34 - 36 and 37 - 38, respectively. The doubling time for cells treated with sodium cyclamate was determined three times. Experiments A, B, and C (Figures 34, 35 and 36) represent the three separate experiments. The doubling time for calcium cyclamate was calculated from two experiments, A and B (Figures 37 and 38). The individual doubling time, average doubling time for each experiment, the overall average doubling time and the standard deviation of the doubling time for each compound and its control are given in

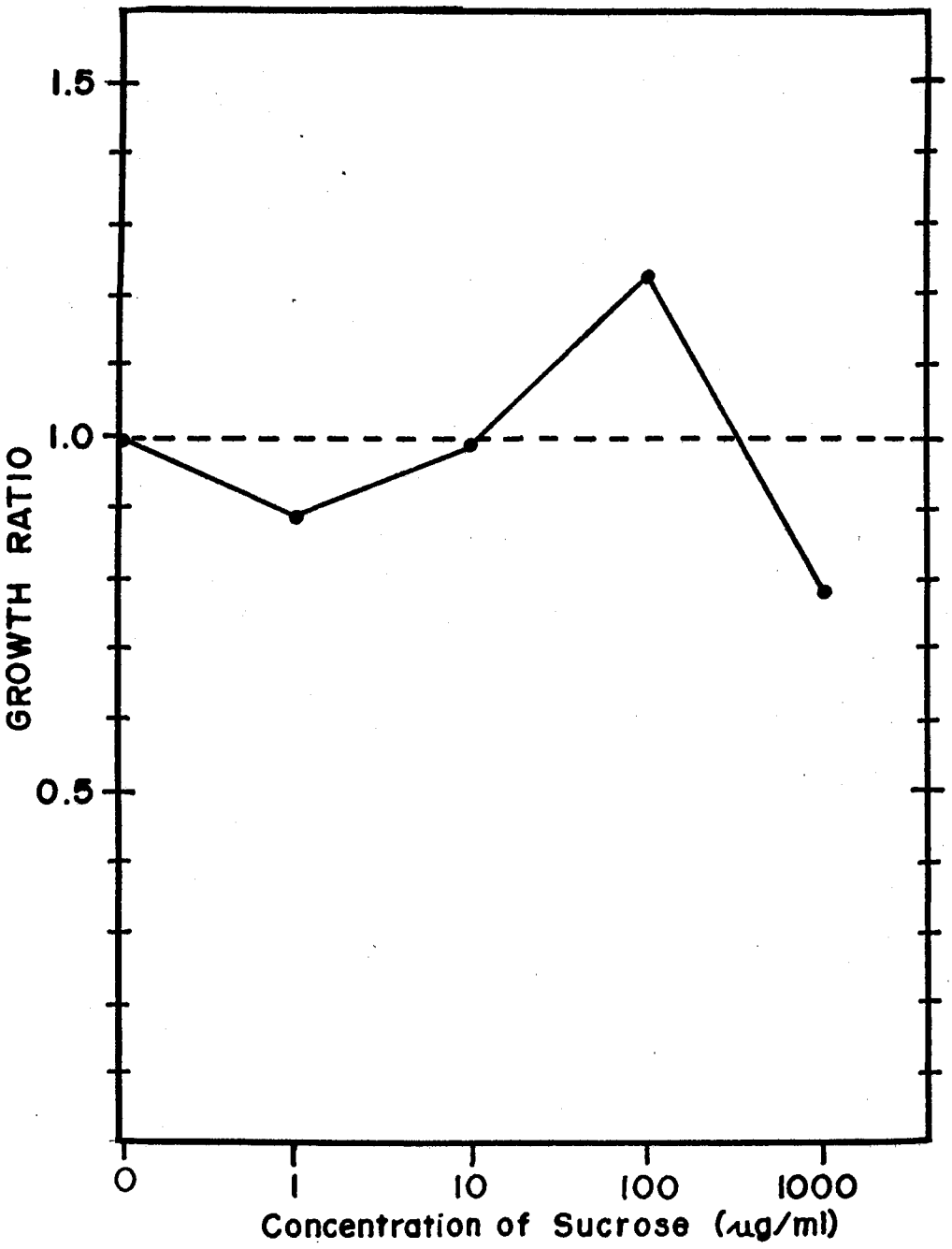

Figure 33. Growth Ratios of Cells after 72 Hours Exposure to Various Concentrations of Sucrose

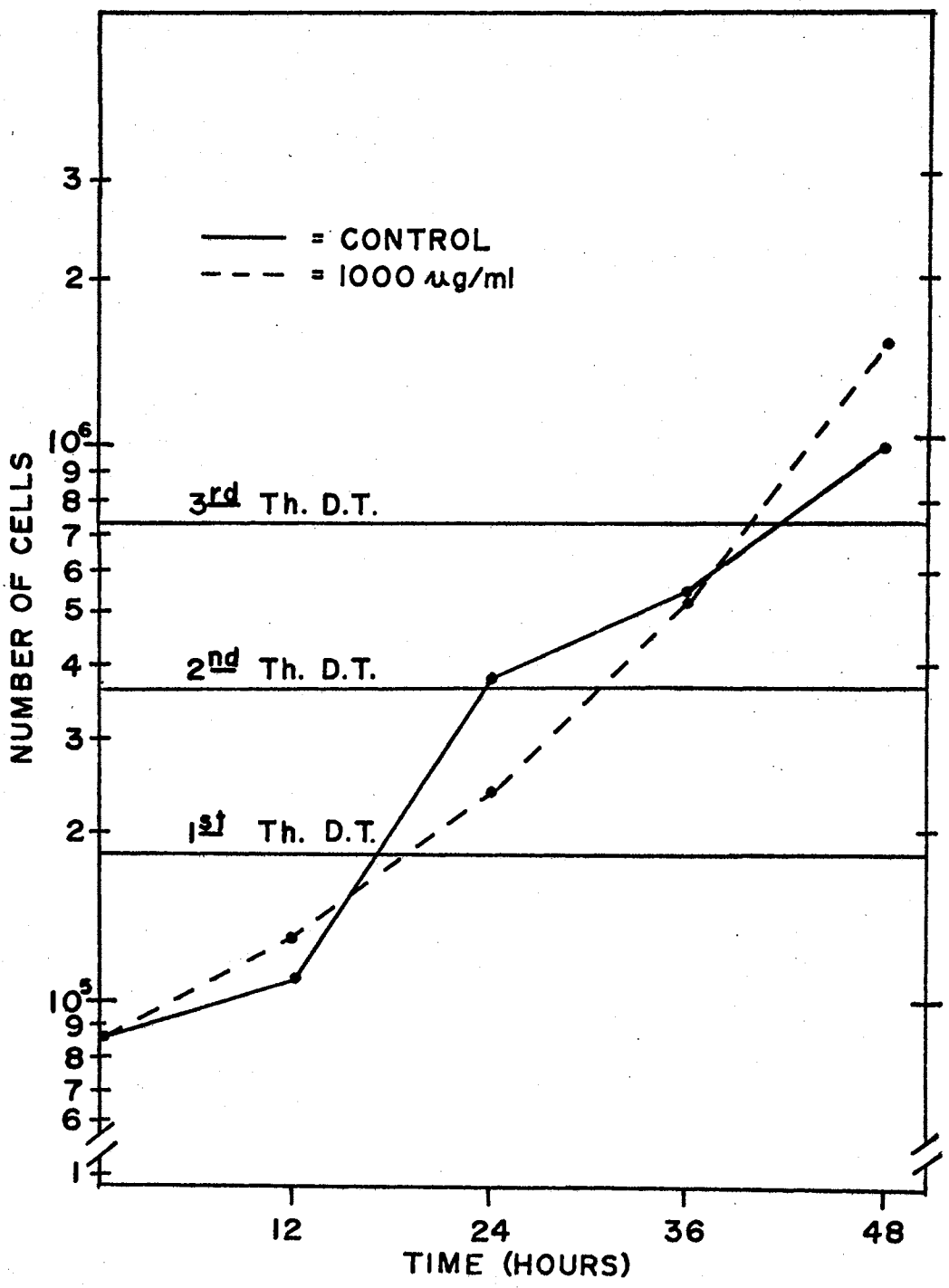

Figure 34. Growth Curve for Cells Exposed to 1000 ug/ml Sodium Cyclamate (Experiment A)

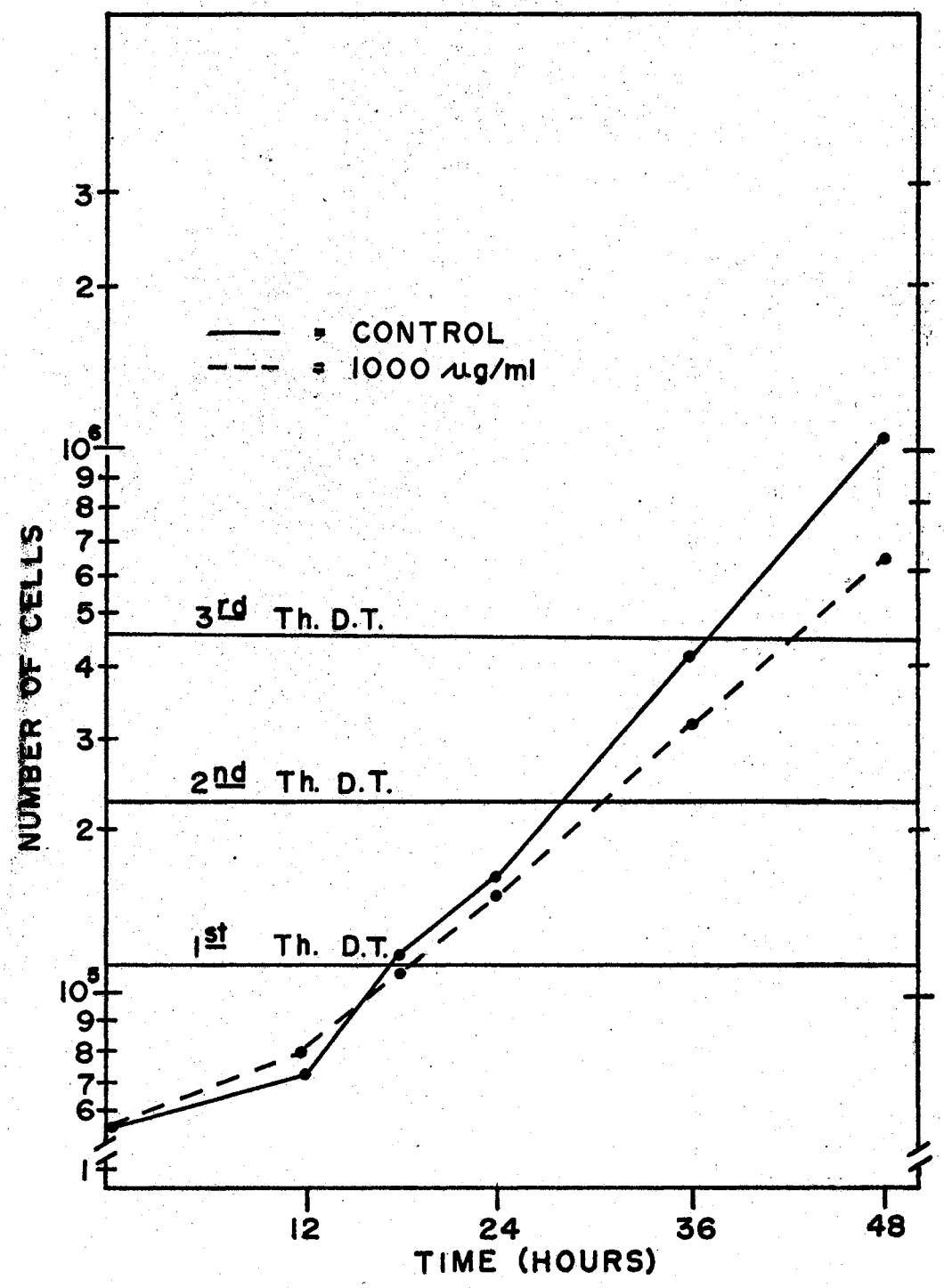

Figure 35. Growth Curve for Cells Exposed to 1000 ug/ml Sodium Cyclamate (Experiment B)

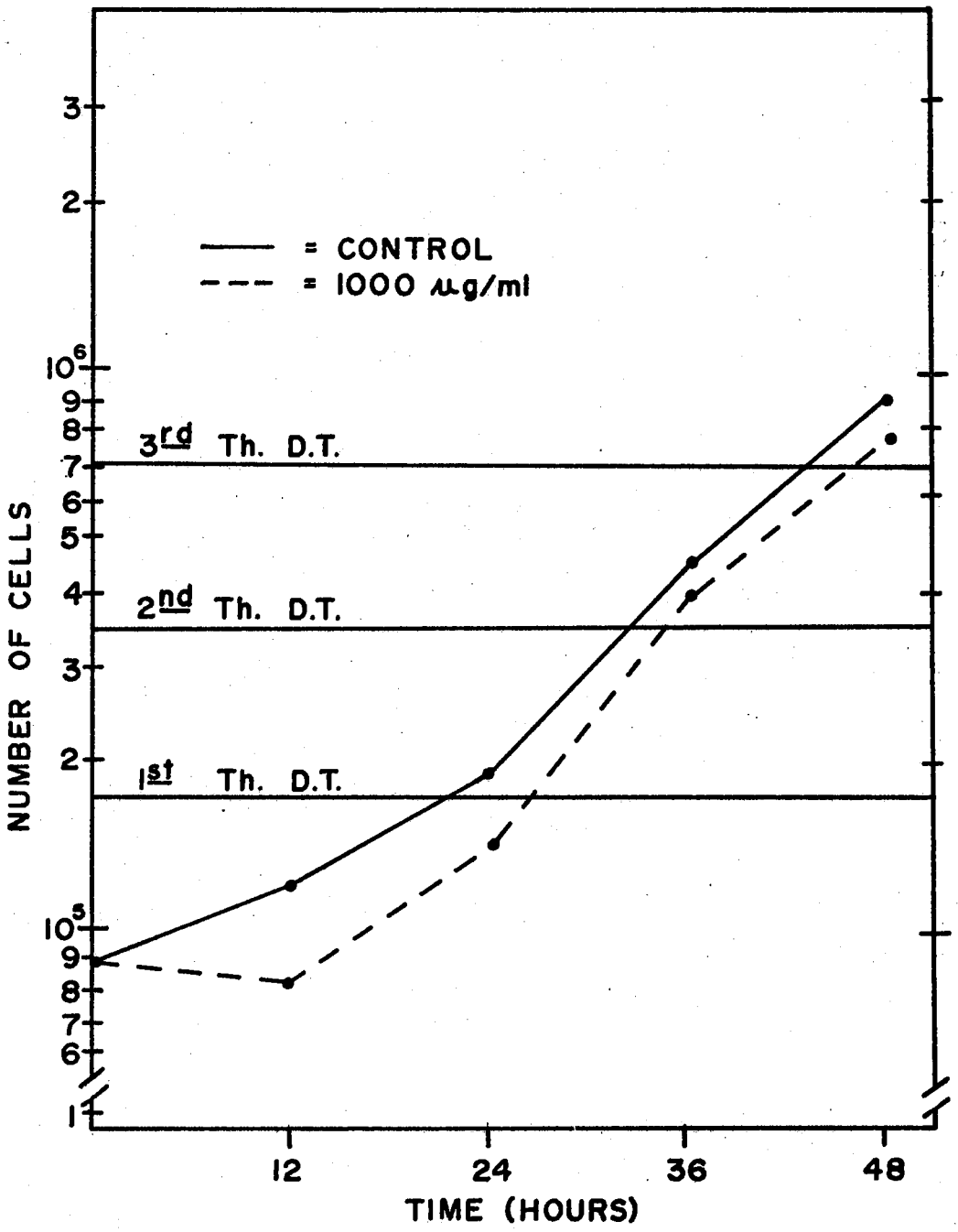

Figure 36. Growth Curve for Cells Exposed to 1000 ug/ml Sodium Cyclamate (Experiment C)

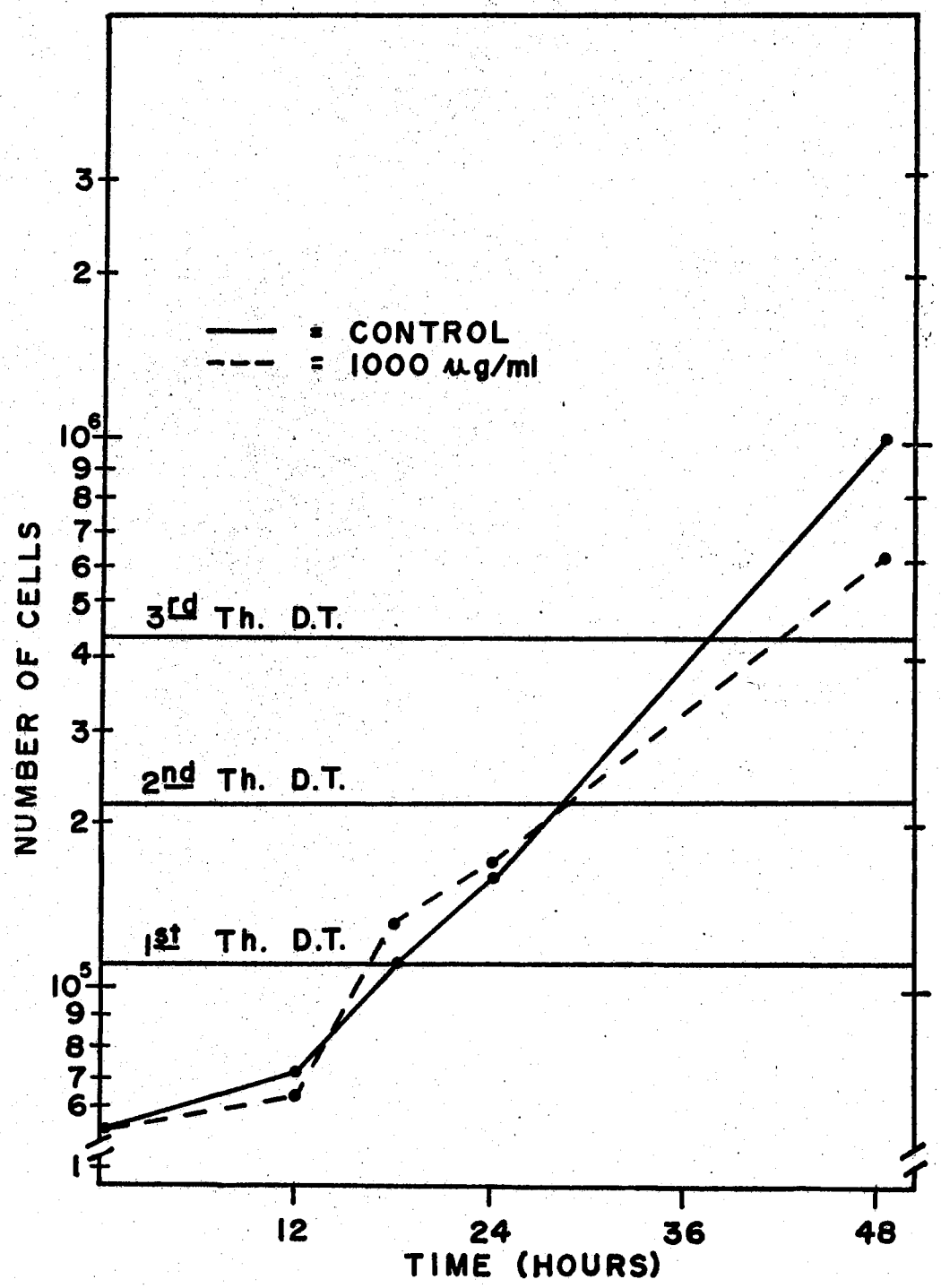

Figure 37. Growth Curve for Cells Exposed to 1000 ug/ml Calcium Cyclamate (Experiment A)

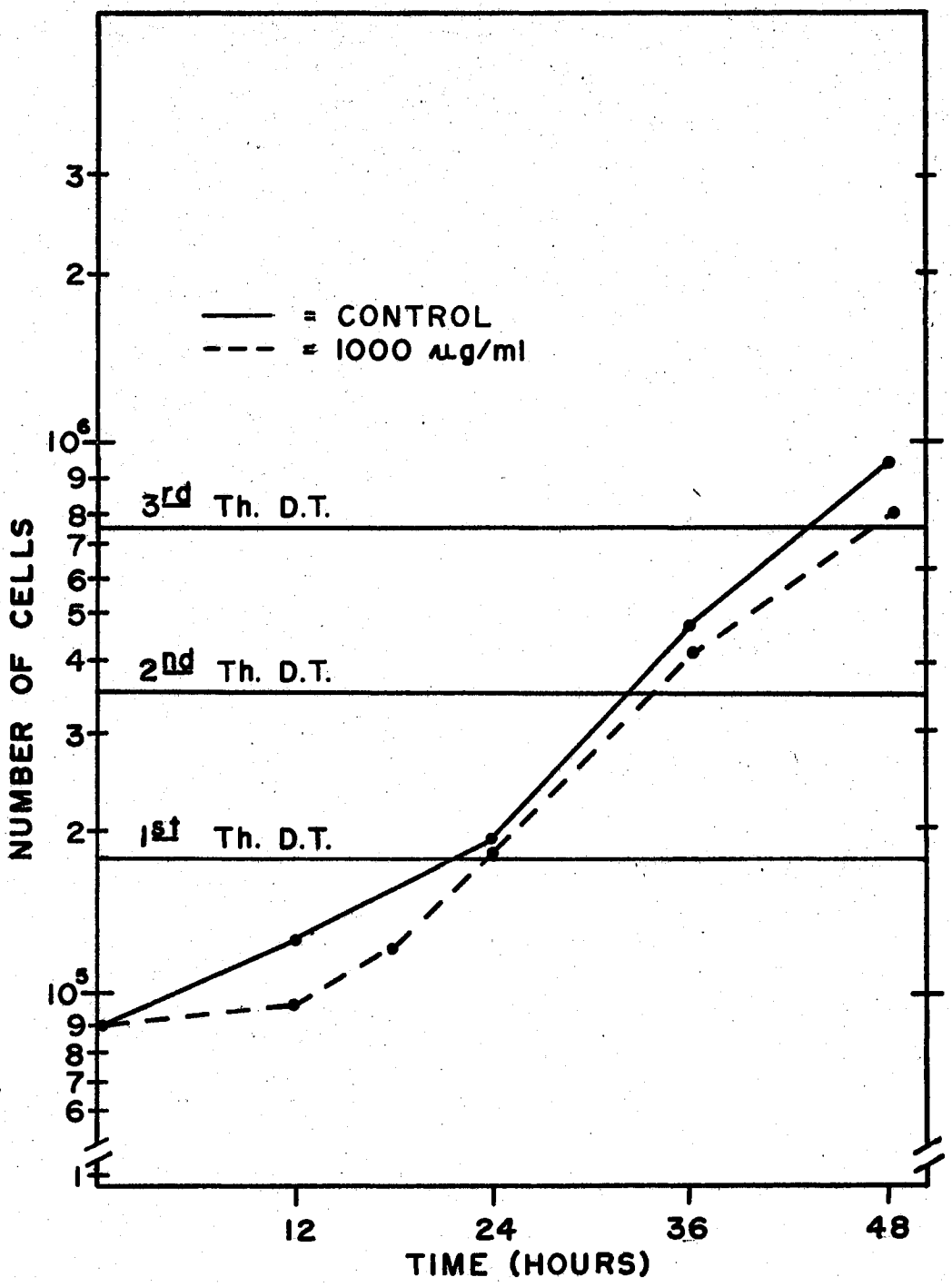

Figure 38. Growth Curve for Cells Exposed to 1000 ug/ml Calcium Cyclamate (Experiment B)

Table XXII. Results of Student's t tests of the null hypothesis that there are no differences between control cultures and cultures exposed to calcium or sodium cyclamate are also given in the table.

It can be observed that considerable variation exists in the time required for a population of cells to double in number. It should be noted, however, that the greatest variation was always seen in the first doubling time for each experiment performed. The mean pooled doubling times of the two controls differ by only 0.32 hour (19.2 minutes). This indicates that the control doubling times are probably reliable although considerable variation does exist between the individual doubling times.

The mean difference between sodium cyclamate and its control is 0.72 hours (43 minutes). The mean difference between calcium cyclamate and its control is 0.74 hours (44 minutes). Thus, the doubling time of cells exposed to sodium cyclamate is one minute faster (compared to the control) than that of cells exposed to calcium cyclamate. By calculation it was shown that control cultures double on the average less than 0.57 hours (34 minutes) faster than cultures exposed to 1000 ug/ml of sodium and 0.15 hours (9 minutes) faster than cultures exposed to 1000 ug/ml of calcium cyclamate.

Analysis of variance using a one way classification and Duncans New Multiple range test indicated that no statistically significant differences existed between the means of any of the four doubling times (Table XXIII).

TABLE XXII

DOUBLING TIMES OF CELLS EXPOSED TO 1000 ug/ml OF SODIUM AND CALCIUM CYCLAMATES

	Dou-bling Number	Doubling Time in Hours			
		Control I	Sodium Cyclamate	Control II	Calcium Cyclamate
Experiment A	1	17.2	18.1	18.0	16.5
	2	16.6	11.9	10.2	13.5
	3	17.6	9.3	8.8	6.9
Average of Doubling Times in Expt. A	$\bar{X}$	13.8	13.1	12.88	12.30
Experiment B	1	17.7	18.5	21.1	23.5
	2	10.4	12.0	10.0	11.5
	3	9.0	11.9	11.2	12.0
Average of Doubling Times in Expt. B	$\bar{X}$	12.36	14.13	14.10	15.60
Experiment C	1	21.4	26.5		
	2	10.9	8.1		
	3	11.0	12.0		
Average of Doubling Times in Expt. C	$\bar{X}$	14.43	15.53		
Mean Pooled Doubling Times of All Expt.	$\bar{X}_G$	13.53	14.25	13.21	13.95
Standard Error of Doubling Times	S.E.	1.67	1.92	1.68	1.87
Difference Between Exptl. and Control - Student's t =		.28 (P 0.7)		.24 (P 0.8)	

TABLE XXIII

ANALYSIS OF VARIANCE FOR DIFFERENCES BETWEEN DOUBLING TIMES OF CULTURES EXPOSED TO 1000 ug/ml SODIUM CYCLAMATE, 1000 ug/ml CALCIUM CYCLAMATE AND CONTROLS

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Sum of Squares	F
Total	29	757.65	26.12	
Between Classes	3	4.22	1.57	0.054
Within Classes	26	752.93	28.95	

$F_{0.05, (3, 26)} = 2.98$

Effects of Long Term Exposures to Low Concentrations (1 and 10 ug/ml) of Sodium Cyclamate on Growth Rate

Although sodium cyclamate, even at concentrations as high as 1000 ug/ml, did not appreciably alter the growth rate of Chinese hamster cells after 72 hours as measured by cell counts, concentrations as low as 1 ug/ml did when cells were exposed over an extended period. These low dosages appeared to have a cumulative effect.

The calculated growth ratio of cultures exposed to 1 ug/ml of sodium cyclamate for 97 days was 0.48. This indicated that these cultures were growing approximately 52% slower than the control cultures. Exposure to 10 ug/ml sodium cyclamate for periods longer than 90 days caused an even greater decrease in growth rate of cells. Cultures grown in the presence of 10 ug/ml for 97, 124, and 134 days

had growth ratios of 0.11, 0.32 and 0.30 respectively (Figure 39). This means that cultures exposed to 10 ug/ml sodium cyclamate on the average, were growing at a rate which was approximately 21% of, or 79% slower than, that of control cultures.

Since these growth rates were determined on cells which had been placed back on normal medium, the effects may be considered "permanent", at least for 48 hours after sodium cyclamate was removed from the medium.

Effects on Later Generations of Cells

The number of cells with chromosome breaks in cultures which had been exposed to 500 ug/ml of sodium cyclamate for 48 hours, then grown on normal medium for 90 days, was greater than the number of cells with breaks in the control group. Seven of 100 cells from the exposed cultures showed breaks, whereas only 2 of 100 had breaks in the control group (Figure 40). This difference was statistically significant at the 0.005 significance level (X^2=12.75; where X^2 .005, 1 dF=7.88). The effects of this 48-hour pulse exposure on chromosome breakage were considered permanent based on the above results (i.e. the breaks were permanent). The distribution of breaks according to type and group of chromosomes affected followed a pattern similar to that previously described under the distribution and frequency of chromosome breaks. The number of pale staining regions in these chromosomes was rather high. Thirty-eight acromatic regions were recorded for the 100 cells examined.

The effects of sodium cyclamate on chromosome number were considered temporary. This conclusion was drawn from the discovery

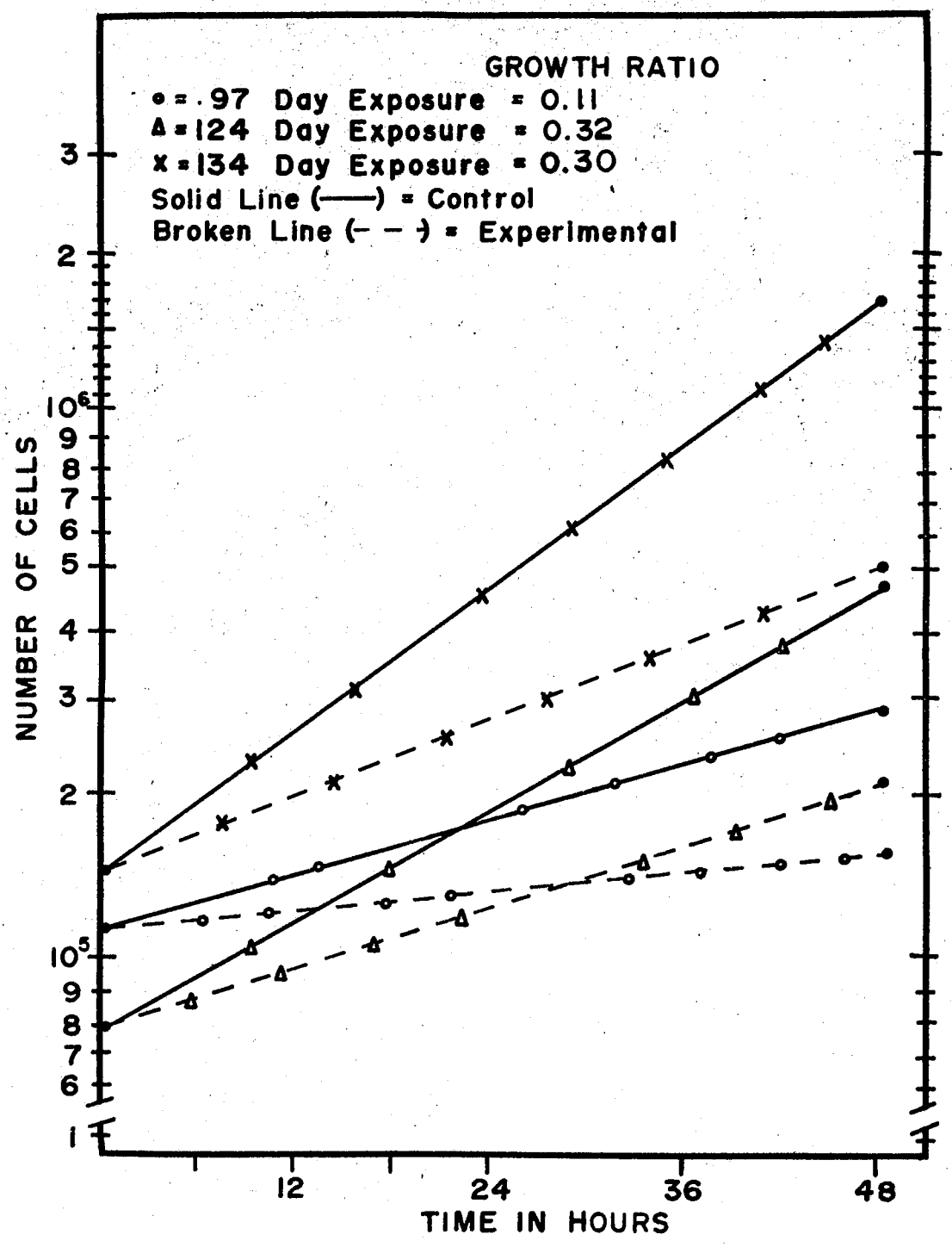

Figure 39. Growth Curves of Cells Exposed to 10 ug/ml Sodium Cyclamates for Periods Longer than 90 Days

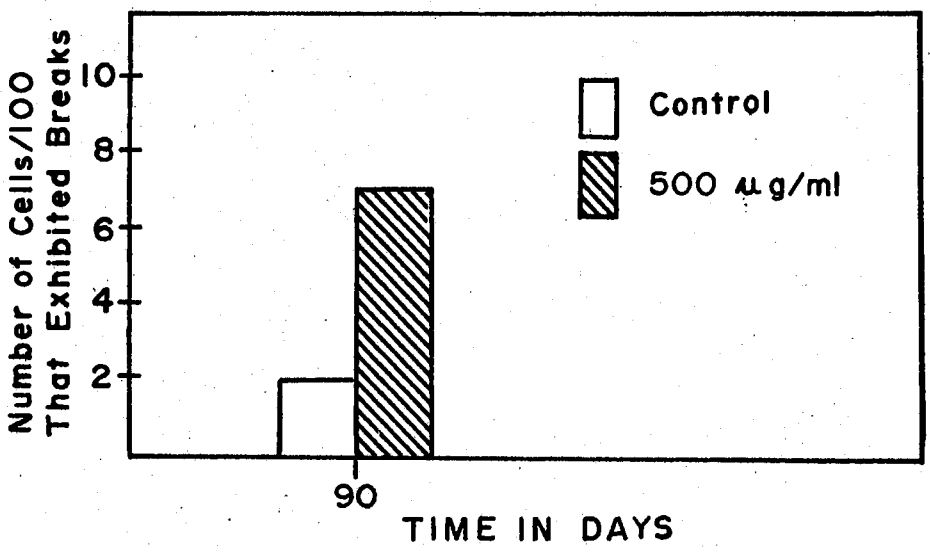

Figure 40. Number of Cells with Breaks after Exposure to 500 ug/ml Sodium Cyclamates for 48 Hours then Grown on Normal Medium for 90 Days

that no differences in chromosome number existed between control cells and cells exposed to 500 ug/ml for 48 hours, then grown on normal medium without cyclamate for 90 days (Table XXIV). It was shown previously that a 72 hour exposure to 500 ug/ml sodium cyclamate caused a statistically significant increase in chromosome number when compared to control cells (Table X).

TABLE XXIV

EFFECTS ON CHROMOSOME NUMBER AFTER EXPOSURE TO 500 ug/ml SODIUM CYCLAMATE FOR 48 HOURS FOLLOWED BY GROWTH ON NORMAL MEDIUM FOR 90 DAYS

	Number of Metaphases						
	20 Group I	20-25 Group II	26-29 Group III	30-50 Group IV	50 Group V	X^2	P
Control	2	86	1	11	0		.500
Exptl.	0	93	0	7	0	5.419	.500

Results of two growth rate experiments performed on cells grown on normal medium for longer than 90 days after a 48 hour exposure to 500 ug/ml of sodium cyclamate showed that the growth rate of these cells was on an average 52% slower than the rate of control cells. The growth ratios from these two experiments, performed 94 and 106 days after the pulse exposure were 0.46 and 0.52, respectively. Figure 41 shows the growth curves for the two experiments.

Inasmuch as a significant change in the growth rate of cells was not detectable at high concentrations after 72 hours exposure in

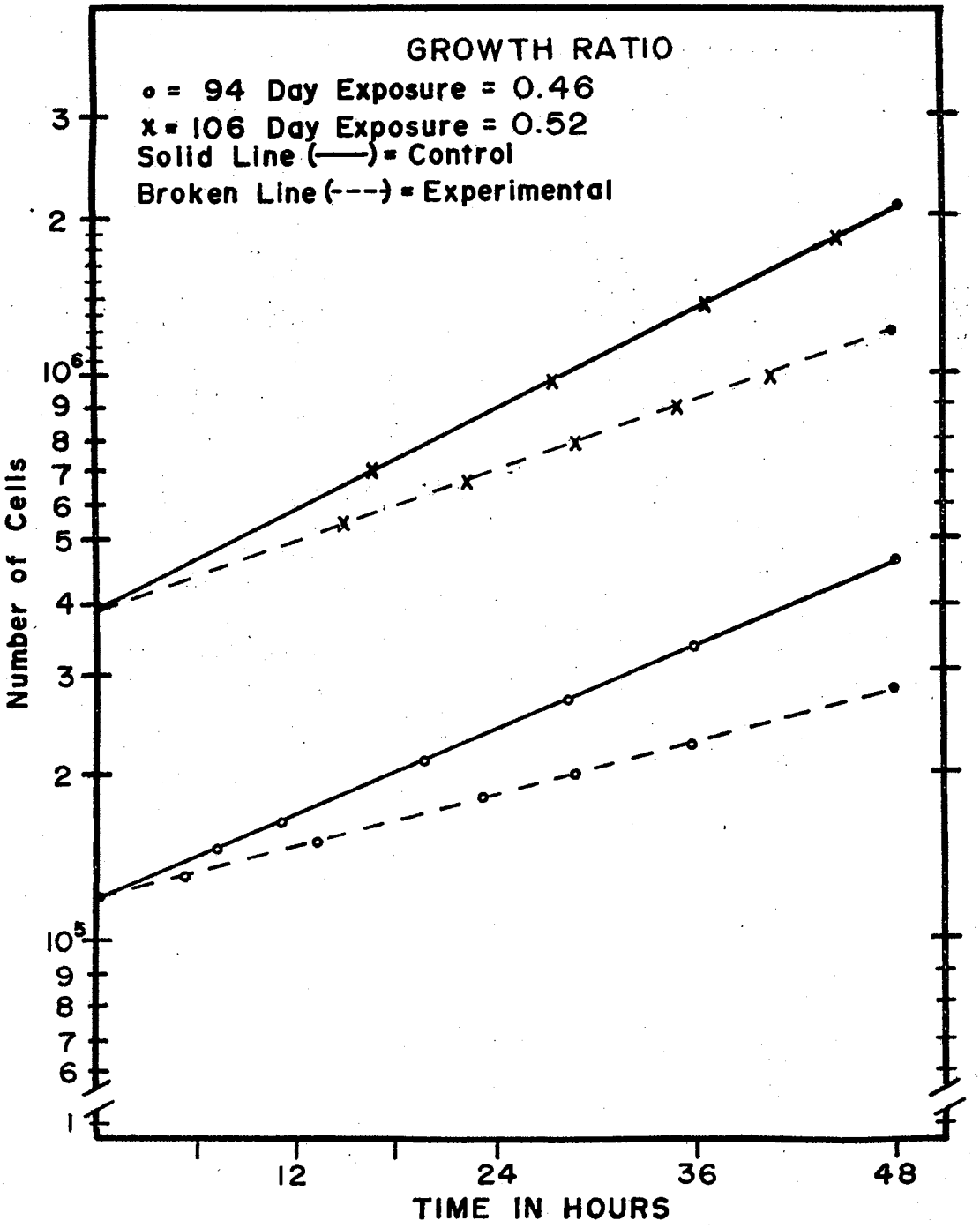

Figure 41. Growth Curves of Cells after Exposure to 500 ug/ml Sodium Cyclamate for 48 Hours Followed by Growth on Normal Medium for Periods Longer than 90 Days

previous experiments but was found in these cells, the effects of cyclamate on growth rate were considered delayed or latent. That the cyclamates have additive effects on growth rate, which are "permanent", was shown previously in experiments using 1 and 10 ug/ml sodium cyclamate.

CHAPTER IV

DISCUSSION

Results of exposing Chinese hamster fibroblasts to different concentrations of the test compounds indicated that only the higher concentrations caused a significant increase in structural and numerical aberrations in cells after a 72 hour exposure period. The minimum concentration required for increasing the number of cells with chromosome breaks significantly was 100 µg/ml for exposure to cyclohexylamine or calcium cyclamate, whereas the minimum for sodium cyclamate was 500 µg/ml. Similar results were reported by Stone et. al., (1969) who found that in vitro a minimum concentration of 250 µg/ml caused an approximate two-fold increase, over the controls, in the number of human leukocytes with broken chromosomes after 72 - 84 hours exposure to calcium and sodium cyclamates.

Stoltz et al., (1970) also found that concentrations of 10^{-3} M (201 µg/ml) sodium cyclamate and 10^{-3} M (99 µg/ml) cyclohexylamine sulfate were required to induce significant chromosomal aberrations in human leukocytes over a 72 hour culture period. Although a slight variation was shown in the above three studies, it appears that concentrations of less than approximately 100 µg/ml of the named compounds fail to cause a significant increase over control cells in the number of cells with chromosome breaks after a 72 hour culture period.

In the present investigation it was observed in the 72 hour

studies that the number of cells with chromosome breaks appeared to decrease after a certain concentration peak was reached. This phenomenon was more clearly shown in exposures to calcium cyclamate than with sodium cyclamate or cyclohexylamine. Figure 6 and Table V show that the greatest number of chromosome breaks was induced by the 100 µg/ml concentration of calcium cyclamate and that concentrations above this level showed progressively fewer cells with breaks than the 100 µg/ml concentration. Figures 1 and 7 show this same phenomenon for cells exposed to sodium cyclamate and cyclohexylamine respectively. A possible explanation for these decreases is that the higher concentrations caused extensive chromosomal damage during the early phases of the culture period and that these cells were damaged to such an extent that they were eliminated from the population at or by the next mitosis. Some validity or support for this assumption is given by Datta and Schleiermacher (1969). Using mice as subjects they found that chromatid aberrations were maximum after a 24 hour treatment with cytoxan (cyclophosphamide) and that the number of damaged cells steadily declined until 96 hours after treatment when no more aberrations could be observed. Schmid and Staiger (1969) achieved similar results with cytoxan in Chinese hamsters. Cytoxan is an alkylating antineoplastic chemotherapeutic agent used in the treatment of certain leukemias. It has been known to cause chromatid breaks and other chromosomal aberrations rather consistently.

The number of cells with breaks after exposure to 10 µg/ml sodium cyclamate for 9 and 62 days and to 1 µg/ml for 97 days was not significantly different from the controls in the present study. However, a 124 day exposure to 10 µg/ml sodium cyclamate produced a highly sig-

nificant number of cells with breaks. Exposure to 100 μg/ml sodium cyclamate for 7 days caused a significant number of breaks and a 60 day exposure caused an even higher number of chromosome breaks and other structural chromosomal aberrations. Since the number of breaks increased with concentration and time, it is proposed that a dose-response relationship exists between the concentration of sodium cyclamate and the number of cells with breaks which is dependent upon time; more time being required for expression of effects in lower concentrations than with higher concentrations. Because a time element was involved, with no other apparent factors being modified, it is further suggested that these effects are probably due to an accumulation of the compound in the cells over long periods of time. Similar dose-response relationships were observed by Cohen et al. (1967), Legator et. al. (1969) and Stoltz et. al., (1969).

It should be noted from Figure 2 that, although not significantly different from its control, the 62 day sample exposure shows an approximate two-fold increase in the number of chromosome breaks (9) over the 72 hour exposure (5). The reason for the non-significance at this time period is that the number of chromosome breaks in the control cells was greater at this time period than at any other sampling period in the study. It was calculated that the mean number of cells with chromosome breaks from eleven different duplicate control cultures was 4.1 per 100 cells. If the 9 chromosome breaks found at the 62 hour exposure period were tested against this mean number, using the Chi-square test, a significant difference would be evident.

Cells exposed to 100 μg/ml of sodium cyclamate for 60 days showed the greatest amount of structural chromosomal damage in the entire

study. Thirty-three breaks were found in 100 cells for this exposure period. The number of exchange figures in all other concentrations and time periods appeared to be randomly distributed, appearing only occasionally. This pattern was broken however, with the 62 day exposure in that four exchange figures that resembled the fusion of an acrocentric and a small metacentric chromosome (Figure 4) and one chromatid exchange that resembled a "quadriradial" were observed. The genetic consequences of these aberrations are not completely clear. However, Kihlman (1966) concluded that "There appears to be a good correlation between mutagenic effect and ability to induce chromosomal aberrations in mammalian cells." Teas, Sax and Sax (1965) indicated that chemical agents which produce chromosome aberrations may also produce cancer in animals. German, Archibald and Bloom (1965) reported that quadriradials and increased chromosomal breakage also characterize the cytogenetic picture in two syndromes; Bloom's syndrome and Fanconi's anemia, which are caused by autosomal recessive genes of low penetrance.

Garriga and Crosby (1959) indicated that the patients with Fanconi's anemia demonstrate an increased frequency of developing neoplasia. They further reported that of the fewer than 70 recorded cases of Fanconi's anemia, leukemia has been found in approximately one-fourth of all the family histories studied. Such exchange figures also are frequently observed in tumor cells that have undergone "malignant transformation" by the oncogenic virus SV_{40} (Hsu and Manna, 1959; Fraccaro et al., 1965; and Wolman et. al., 1964).

Results of tabulating and categorizing chromosome breaks with cells exposed to various concentrations of sodium cyclamate indicated

a non-random distribution of breaks. The majority of breaks were found in chromosome number one. Certain areas of the long arm of this chromosome seemed more susceptible to breaks than others. Cohen and Shaw (1965) demonstrated "hot spots" in the heterochromatic regions of chromosome number one in man. Intrachromosomal localization of chromosomal breaks in chromosome number one have been induced by many agents: herpes-simples virus (Hampar and Ellison, 1961 and 1963); measles virus (Nichols et al., 1964); 5-bromodeoxyuridine (Hsu and Somers, 1961); and lysergic acid diethylamide (Cohen et al., 1967).

Chromosome breaks resulting from exposure to a high concentration of sodium cyclamate were concluded to be permanent on the basis of the manner of analysis described in Chapter II (Materials and Methods). It should be pointed out that this conclusion was drawn from a comparison of exposed cells with control cells that showed only two cells with breaks per 100 cells. Since this number of breaks was below the mean number of breaks in control cells (4.1/100 cells) the validity of the above conclusion may be questionable. Nevertheless, the number of pale staining chromosomal regions was greater in these cultures than in any other cultures studied. This observation may indicate greater genetic consequences than large visible breaks.

Sucrose in concentrations of 1, 10, 100 and 1000 μg/ml failed to cause an increased number of cells with chromosome breaks over control cells after a 72 hour exposure period. It should be noted that concentrations of 250 and 500 μg/ml were not studied for this compound. Based on results from this study on the cyclamates, and also results of other investigators, the concentration range in which significant chromosome breaks are induced is between 100 and 1000 μg/ml. Chromo-

some breakage studies using concentrations of 250 and 500 μg/ml sucrose need to be performed before a definite conclusion is drawn that sucrose does not cause chromosomal damage to Chinese hamster fibroblasts.

Cells exposed to high concentrations of sodium cyclamate (500 and 1000 μg/ml) and cyclohexylamine (250 μg/ml) showed a statistically significant different chromosome number from that found in control cells not exposed to these compounds. Cyclohexylamine was considered to have a greater effect on chromosome number than sodium cyclamate since it caused a greater deviation from the control than sodium cyclamate and did so at a lower concentration. The shift of chromosome number was from the approximately diploid condition to a triploid to tetraploid condition under the influence of sodium cyclamate. Under the influence of cyclohexylamine the shift from the approximately diploid state was nearly equally distributed between a hypo-ploid and the triploid to tetraploid condition. A comparison of these results with those of other investigations using cyclamate or cyclamate-related compounds was not made since comparative data were not found in the literature. However, Sparkes et al., (1968) demonstrated that lysergic acid diethylamide which caused increased chromosomal breaks also caused changes in chromosome number in human leukocyte cultures. Kihlman and Levan (1949) reported that caffeine prevents cell wall formation in Allium root tips which leads to the formation of cells with high numbers of chromosomes after successive mitoses. In our laboratories Cook (1972) found that urethan (ethyl carbamate) in concentrations as low as 300 μg/ml caused a significant increase in the chromosome number of Chinese hamster fibroblasts after a culture period as short as six hours, in vitro.

The growth rate of Chinese hamster fibroblasts exposed to various concentrations of the test compounds was ascertained by three methods in this study: (a) determination of a growth ratio between control and experimental cultures after a specified time period by cell counts using a Coulter counter, (b) calculation of doubling time of cell populations and (c) by the metaphase index method. Results of all three methods indicated slight decreases in growth which were concentration dependent when cells were exposed to sodium or calcium cyclamate at concentrations up to 1000 μg/ml.

Sodium cyclamate in concentrations of 5000 and 10,000 μg/ml significantly reduced growth rate. Exposures to 100,000 μg/ml completely inhibited growth. Cells exposed to the latter concentration appeared granular and vacuolated.

Sucrose at a concentration of 100 μg/ml stimulated the growth of cells over a 72 hour period whereas at 1000 μg/ml it caused a decrease as measured by the growth ratio and the metaphase index method. The decrease was 23% of the control rate as measured by growth ratio and 10% as measured by the metaphase index.

Microscopic examination of attached cells after 72 hours exposure to 1000 μg/ml sucrose revealed that many cells were circular in shape and did not appear as glossy and healthy as control cells. Vacuoles in the cytoplasm were not evident at this concentration.

The decreases in growth at the higher concentrations of sodium cyclamate and for sucrose agree with the findings of Bernacki and Bosmann (1971). Using a doubling time procedure, almost identical to that described in the present investigation, they found that control cultures of murine $L_{5178}Y$ leucocytes had a doubling time of 11.5 hours

while cultures exposed to 0.05 M (17,100 μg/ml) sucrose had a doubling time of 14 hours, a 0.08 M (27,360 μg/ml) culture for 22 hours. A concentration of 0.2 M sucrose was found to be toxic and caused lysis. They concluded that an inverse relationship exists between the concentration of sucrose and growth and suggested that growth rate is related to the final osmolarity of the medium. In experiments using sodium chloride they found that the doubling time was 18 hours for exposure to concentrations of 0.025 M (1,450 μg/ml) and that 0.05 M (3,900 μg/ml) sodium chloride caused a zero growth rate. The effect of sodium cyclamate on the doubling time was also reported. This salt was considered toxic at a 0.025 M (5,025 μg/ml) concentration. They especially pointed out that whereas sodium cyclamate completely inhibited growth at this concentration, sodium chloride only depressed growth. It is the thinking of the present investigator that if osmotic effects are involved this is what is expected and a better comparison would be made by using equivalent weight units instead of molarity.

Bernacki and Bosmann (1971) also reported that after a 48 hour incubation period in 0.08 M sucrose there was a slight decrease in cell size of a magnitude similar to that found in hypertonic sodium chloride. Similar changes in morphology were observed in cells exposed to high concentrations of sucrose (1000 μg/ml) and sodium cyclamate (10^4 μg/ml) in the present investigation. Cells were rounded and had lost their glistening appearance.

The development of vacuoles in cells exposed to hypertonic solutions of sucrose has been studied *in vivo* and *in vitro* in a number of animals and cell culture systems. These solutions have been shown to

influence growth rate and enzyme activity. Munro (1968) reported that Mullendorf in 1936 demonstrated that rabbit fibroblasts cultured in natural medium made hypertonic with sucrose became filled with vacuoles. Brewer and Heath (1964) suggested that these vacuoles develop from vacuoles in lysosomes. Dingle, Fell and Glauert (1969) noted that in organ cultures of bone and cartilage, vacuolation was induced by sucrose and was associated with an increased synthesis of lysosomal enzymes. Munro (1968) found that the addition of 0.08 M sucrose (27,360 µg/ml) to the culture medium caused vacuolation of Chinese hamster fibroblasts. He concluded that the vacuoles were formed by pinocytosis rather than the swelling of lysosomes and that the extreme vacuolation exhibited by cells after a number of hours exposure was due to the fibroblasts' inability to digest the hypertonic sucrose in the vacuoles; hence these vesicles tend to expand osmotically.

Benassi (1968) found that mouse cells, exposed to 0.08 M sucrose showed decreased growth rate, increased vacuolation and increased acid phosphatase and lysosomal enzyme production. He concluded that the development of vacuoles and the increased enzyme activity seem to be connected and are probably responsible for the decreased rate of cell proliferation. De Duve and Wattiaux (1966) described a relationship between vacuolation and the release of lysosomal enzymes with resulting breakdown of intercellular substance.

The highly significant decreases in growth rate after long periods of exposure to low concentrations of sodium cyclamate reported here may be caused by an accumulation of the compound in the cells leading to decreases in growth rate by similar mechanisms as described by Munro (1968), Benassi (1968) or Dingle et al. (1969) for sucrose.

Another possible explanation for the delayed decrease in growth is that long periods of time are required to accumulate enough cyclohexylamine from the breakdown of cyclamate to cause expression of toxic effects that lead to decreases in growth. It may take several days (e.g., 33 or at least more than 9, Figure 26) to convert enough of the low concentrations of cyclamate into cyclohexylamine to cause significant effects on growth.

Cyclohexylamine caused a significant decrease in the growth rate of Chinese hamster fibroblasts at concentrations as low as 1 μg/ml after a 72 hours exposure period. Cell division was completely inhibited when cells were exposed to 500 μg/ml and a 1000 μg/ml concentration was considered lethal (Figure 28). Since cyclohexylamine is considered toxic (Wiegand, 1969; Biava, 1969; Kojima and Ichibagase, 1966) the above results were not unexpected.

It appears from Figure 26 that length of exposure to 10 μg/ml sodium cyclamte does not cause a linear decrease in the number of metaphases with time. A large variation in the number of metaphases from one period to the next is evident and recognized. A possible explanation for the large number of metaphases (in both control and experimental cultures) at one time period and a low number of metaphases at others is that the slides were prepared when cells were at different phases of the mitotic (cell) cycle. At certain phases of the cycle, especially if cells have a high degree of synchrony, the number of metaphases would be very abundant (around metaphase of mitosis) and at other phases the number of metaphases would be lower. If, for example, the three hour colcemid treatment were applied approximately three hours before the synchronized cells were approaching

the metaphase stage of cell division, a large number of metaphases would accumulate. Whereas if this synchronized population of cells were treated with colcemid just after a cell division, the number of metaphases would be lower than in the previous case. No determination was made of the degree of synchrony of cell division in the cultures.

CHAPTER V

SUMMARY AND CONCLUSIONS

The *in vitro* effects of sodium cyclamate, calcium cyclamate, cyclohexylamine and sucrose on growth rate, chromosome breakage and chromosome number were studied using Chinese hamster fibroblasts.

Cells were grown as monolayers in McCoy's 5A medium supplemented with 10% fetal calf serum with or without various concentrations of the test compounds for various periods of time.

Growth rates were determined by two methods: cell counts with the Coulter counter, and by use of the metaphase index. Structural and numerical chromosomal aberrations were studied in 50 or 100 metaphase spreads from slides prepared by a colcemid-hypotonic method and stained with Giemsa.

Sodium Cyclamate

Exposure to sodium cyclamate at high concentrations for a 72 hour period caused a significant increase in the number of chromosome breaks and in the chromosome number of Chinese hamster fibroblasts. Concentrations below 500 μg/ml did not cause these increases. The rate of cell division as determined by the metaphase index method was decreased at high concentrations (250, 500 and 1000 μg/ml) of this compound. The growth rate of cells as measured by cell counts after 72 hours exposure to 100 and 1000 μg/ml was slightly less than (10

and 14% respectively) but not statistically different from the growth rate of control cells. The average doubling time of cells exposed to 1000 μg/ml, studied over a 48 hour period, was 34 minutes slower than the doubling time of control cells. This difference was not statistically significant. The average doubling time for control cells was calculated at 13.5 hours, which is close to that found by other investigators.

Sodium cyclamate, when used to treat cells at a concentration of 10 μg/ml for 9 and 62 days, did not cause an increase in the number of cells with chromosome breaks over that of the control. However, after 124 days exposure to this concentration a highly significant increase (0.005 level) in the number of cells with chromosome breaks was observed. A significant increase in chromosome number was also observed at this concentration at all of the above sampling periods. The growth rate of cells was significantly slower than that of control cells after exposure to 10 μg/ml for 33, 62, 90 and 124 days as measured by the metaphase index. However, it was slightly (not significant) faster after only 9 days exposure to the same concentration. When measured with the Coulter counter, cells exposed to 10 μg/ml for periods longer than 90 days showed a growth rate 79% slower than that of control cells.

Cells exposed to 1 μg/ml for 97 days in vitro did not show a significant increase in chromosome breaks or chromosome number over control cells. However, the growth rate was decreased by 52% as measured by cells counts for this exposure period.

Although exposing cells to 100 μg/ml for 72 hours (3 days) did not cause a significant increase in cells with chromosome breaks,

exposure periods as short as seven days showed an increase in chromosome breaks (significant at the 0.005 level). Cells exposed to this concentration for 60 days showed an even greater number of breaks than after the seven day exposure. Changes in chromosome number were not observed after 60 days exposure to 100 μg/ml; however, an increase in chromosome number, significant at the 0.05 level, was observed in cells exposed to sodium cyclamate for the seven day period. The majority of chromosome breaks were of the chromatid type and were found in the long arm of chromosome number one.

Based on results of exposing cells to 500 μg/ml for 48 hours, then growing them on medium without cyclamate for 90 days, the chromosome breaks were considered permanent, whereas the changes in chromosome number after exposure to 500 μg/ml were considered temporary. The *in vitro* effects on growth rate were considered to be latent on the same basis as given above. It was thus concluded that: (a) only high concentrations cause changes in growth rate and significant chromosomal aberrations after a 72 hour exposure to sodium cyclamate, (b) exposures to low concentrations for extended periods cause an increase in chromosome number and the number of cells with chromosome breaks, and a relationship exists between concentration and response which appears to be time dependent, (c) long term exposures to low concentrations decreases the growth rate of cells "permanently", and (d) the decreases in growth rate and the observed chromosomal aberrations are achieved in cumulative effects over long periods of exposure to the low concentrations (1 and 10 μg/ml) of sodium cyclamate.

Calcium Cyclamate

Results of observations on 50 metaphases per concentration from cells exposed to 1, 10, 100, 250, 500 and 1000 µg/ml of calcium cyclamate showed that only one concentration, 100 µg/ml, produced a greater percentage of cells with chromosome breaks than control cells. Chromosome number and growth rate as measured by cell counts were considered unaffected over the 72 hour exposure period. The average doubling time of cells exposed to 1000 µg/ml was only 0.15 hours (9 minutes) slower than the average doubling time of control cells. This difference was not significant. However, growth rate as measured by the metaphase index for cells exposed to high concentrations (250, 500, and 1000 µg/ml) over the 72 hour exposure period was significantly (0.05 level and below) slower than the growth rate of control cells.

Calcium cyclamate differs from sodium cyclamate in that it did not induce changes in chromosome number over a 72 hour exposure period. Chromosome breaks were effected at a lower concentration (100 µg/ml) with calcium than with sodium cyclamate (500 µg/ml).

Calcium and sodium cyclamates showed similarities in their effects on growth in that: (a) only the three highest concentrations studied (250, 500 and 1000 µg/ml) for both compounds caused decreases in growth rate as measured by the metaphase index, (b) neither compound caused significant changes in growth rate of cells as measured by cell counts and (c) the difference in doubling times of cells exposed to these two compounds was only one minute.

Cells exposed to 100 and 250 µg/ml of cyclohexylamine for 72 hours showed a significantly higher percentage of chromosome breaks

than control cells. Concentrations less than 250 µg/ml did not produce more breaks than found in controls. The only concentration of this compound to cause a significant change in chromosome number from that of the control was 250 µg/ml (significant at the 0.001 level). Only 24% of the 50 cells counted showed the "normal" diploid complement of chromosomes at this concentration. Chromosome breakage and number were not studied for cells exposed to 500 and 1000 µg/ml of cyclohexylamine. The 500 µg/ml concentration completely inhibited cell division and 1000 µg/ml was lethal. Growth rate, as measured by cell counts using the growth ratio (CCNSC) was unaffected by a 72 hour exposure to 10 µg/ml. However, concentrations of 100 and 1000 µg/ml decreased growth rate by 56 and 99.6% respectively. All concentrations (1, 10, 100, 1000 µg/ml) studied showed a significant decrease in growth rate as measured by the metaphase index method after 72 hours of exposure to this compound.

It was concluded that the breakdown product of cyclamate, cyclohexylamine, was more effective in decreasing growth rate over a 72 hour period than the cyclamates. Its effects on chromosome number and chromosome breakage were only slightly different from those shown by the cyclamates.

Sucrose

Results of exposing cells to concentrations of sucrose as high as 1000 µg/ml for 72 hours indicated that this compound was not effective in causing a significant increase in cells with chromosome breaks or alterations in chromosome number. The growth rate of cells exposed to 1, 10 and 100 µg/ml was faster than the growth rate of

control cells as measured by the metaphase index. These three concentrations were not significantly different from the control when measured by cell counts after 72 hours exposure to the compound. Exposure to 1000 µg/ml for 72 hours decreased the growth rate of cells by 23% as measured by cell counts and by approximately 10% as measured by the metaphase index. This depression of growth was concluded to have resulted primarily from the increased osmolarity of the medium.

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VITA

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