ANTIMITOTIC EFFECTS OF TERRAMYCIN

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Stella Marie Wilson

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This is to certify that the

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Antimitotic Effects of Terramycin

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Stella Marie Wilson

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~~Masters~~ degree in Botany (cytology)

Major professor

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ANTIMITOTIC EFFECTS OF TERRAMYCIN

By

Stella Marie Wilson

AN ABSTRACT

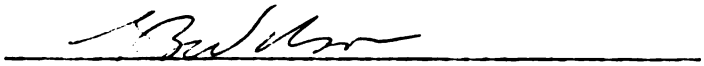
Submitted to the School of Graduate Studies of Michigan State College of Agriculture and Applied Science in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Department of Botany and Plant Pathology

School of Science and Arts

Year 1954

Approved by 

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Stella Marie Wilson

The Pisum test was used to determine the cytological effects of terramycin-HCl. The root tips of young seedlings of Pisum sativum were treated for eight hours with concentrations of 10 ppm. to 200 pm.; and for short term treatments of 15 minutes with 100 and 200 ppm. and for 30 minutes with 600 and 800 ppm. followed by a recovery period in nutrient solution. A quarter-strength modified Hoagland solution was used as solvent. Cuttings were made at the beginning of each run and at spaced intervals thereafter. Slides were made using the Feulgen squash technique and were microscopically examined and scored.

It was observed that the mitotic index decreased with increase in time and concentration. Early prophases disappeared. Late prophase showed an increase in proportion to total mitotic cells and was characterized by contracted figures and reversions. Post prophases were the least effected. Abnormalities were of the "spread" and "scattered" types.

It was concluded that: 1) terramycin-HCl has an inhibitory effect on "antephase"; 2) interferes with center and kinetochore movement in prophases; 3) may have a slight destructive effect on the spindle; 4) prevents cells in mitosis at the time of treatment from dividing again. No mutagenic action was indicated for this antibiotic.

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Terramycin-HCl differs from colchicine in respect to cytological effects: 1) the former inhibits "antephase," whereas, the latter stimulates "antephase"; 2) the former does not have much effect on post prophase, whereas, the latter does; 3) the former inhibits effected mitotic cells from dividing again, whereas, the latter does not.

The cytological effects of terramycin-HCl does not differ from those of other antibiotics studied thus far. There is some indication of a correlation between cytological effects and antibiotic action of the antibiotics.

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_____ and C. C. Bowen. 1951. Cytological effects of some more antibiotics. Jour. Hered. 42:251-255.

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ACKNOWLEDGEMENTS

The writer wishes to extend her sincere thanks to Dr. G. B. Wilson, under whose direction this research was conducted. His guidance and patience will long be remembered.

To my colleagues, thanks are extended for their friendship and aid. To Mr. Philip G. Coleman, is extended thanks for his photographic work.

Appreciation is extended to the All College Research Committee for defraying many of the research expenses, and also to the Naval Research Grant Assistantship which made it possible for the writer to conduct this study.

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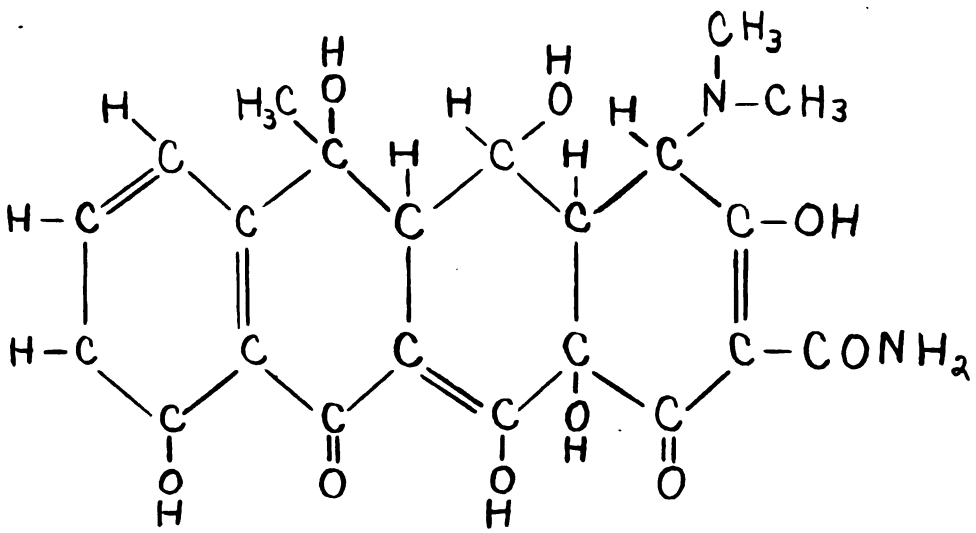
INTRODUCTION

The primary purpose of the research for this thesis was to determine the antimitotic effects of terramycin. A preliminary investigation of the effects of this antibiotic was made by G. B. Wilson and C. C. Bowen (1951), using the **Allium cepa** test. At that time, it was determined that the general mode of effect was the same as of aureomycin, actidione and streptomycin, but a detailed analysis was not made.

Terramycin was introduced as an antibiotic in 1950. It is produced by Streptomyces rimosus. The structural formula of terramycin is as follows (from Peterson and Strong, 1953):



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Pure terramycin is highly insoluble and is amphoteric in form. However, the sodium and hydrochloride salts of terramycin are readily soluble. The dry hydrochloride salt is very stable and shows no loss of activity after prolonged storage at 25°C.

Terramycin and its salts have a broad antimicrobial spectrum and are effective against aerobic and anaerobic, Gram-positive and Gram-negative bacteria and certain of the rickettsiae. They have been found to be a very effective treatment of some virus diseases, pneumonia, and certain venereal diseases; and have shown some promise of tuberculostatic activity.

Terramycin is readily absorbed by the blood stream following oral or paranterial administration. Complete elimination in the urine is realized within one to two days, depending upon the quantity administered. It is very similar to aureomycin in this respect. Terramycin and its salts have been generally accepted as being a highly effective chemotherapeutic agent, with very little toxic effect.

In addition to its medical uses, terramycin (mainly the hydrochloride salt) has been used in treatment of animals and plants. The addition of terramycin - HCl along with vitamin B_{12} to the diets of turkey poults, chicks, hogs and other domestic animals has had favorable growth stimulating effects. It has also been found to decrease effectively the intestinal microflora of hogs, calves and turkeys.

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L. G. Nickell (1953) has found that treatment of seeds of cucumber, corn and narrow-leaved sorrel, with concentrations below 25 ppm. added to the soil, increases germination percentage and growth rate. In contrast to these results there is a report from Italy by D. Della Bella and C. Gabellini (1951), of reduced root growth of Lupinus albus when treated with 10 ppm. in aqueous solution. Also, from Brazil by M. Mateus Ventura (1952) comes a report of markedly inhibited growth of roots of Zea mays. However, in this case the higher concentrations of 50 to 1500 ppm. were used.

Viewing the wide scope and use of terramycin, it seemed warranted to make a more complete analysis of the cytological effects using the Pisum test. (C. C. Bowen and G. B. Wilson, 1954).

LITERATURE REVIEW

The first report of cytological effects of antibiotics was made by G. B. Wilson (1950). The antibiotics Penicillin G, Streptomycin, Neomycin, Circulin, Endomycin, Actidione and Streptothricin were investigated using the Allium test. Penicillin G and Endomycin showed very little measurable effects; Neomycin and Circulin showed few mitotic deviations, but were highly toxic; the others showed "C-mitotic" effects at the toxic level. In all cases, some "reductional groupings" were found. In 1951, Wilson and Bowen reported on the cytological effects of Aureomycin, Terramycin, Streptomycin and Chloromycetin on Allium root tips. All were capable of inducing mitotic aberrations of the "C-mitotic" type. Cytological and toxicity thresholds were close and recovery was not realized, with the exception of Chloromycetin, in which recovered cells were normal.

Levan and Tjio reported on Penicillin in 1951, in which they used the Allium test and concentrations ranging from 0.[illegible]1 to 20 MIU (million international units). The stronger concentrations of 10 to 20 MIU induced lethality. "C-tumour" was produced by concentrations of 0.[illegible] to 1 MIU, and "C-mitosis" by 2 to 5 MIU. In addition, a low and erratic frequency of radiomimetic effects (pseudo-chiasmata, attached and free

fragments, and stickiness) was reported. From these results, they concluded that Penicillin had some, but very weak, mutagenic activity.

Hawthorne and Wilson (1952) reported on a further investigation of Actidione. Effects were: a decrease in mitotic activity correlated with time; fluctuation in the relative frequencies of stages correlated with time and concentration; toxicity at concentrations producing "C-mitosis"; and general effects of contraction, "split" figures, and stickiness. Tanaka and Satô (1952), reported an investigation of the effects of Streptomycin as observed on Tradescantia paludosa, using the concentrations of 25 ppm. to 1000 ppm. Effects reported were: contraction, "C-mitosis", stickiness, fragmentation of the bridge and fragment type, micro-nuclei, faint-staining prophase chromosomes, and clotting at the sub-lethal range. They concluded that the action of Streptomycin was mutagenic.

Bowen and Wilson (1954) reported a further investigation on the cytological effects of Actidione, Streptomycin and Chloromycetin, using the Pisum test. Actidione was determined to fulfill the D'Amato requirements of a "prophase poison." Scattered metaphases were not considered to be "C-mitotic" and the term "akinetic mitosis" was proposed to describe them. Streptomycin was similar in effect to Actidione, with very little margin between cytological and toxic concentrations.

Chloromycetin was the least toxic, exhibiting preprophase inhibition of mitosis at sublethal levels.

In recent years, there has been some controversy on the terminology used to describe cytological effects. "C-mitosis" was the term used by Levan (1938) to describe the modification of the mitotic cycle by colchicine. In its strictest sense, "C-mitosis" includes spindle suppression, and a delay in kinetochore cleavage. "C-mitotic" substances are polyploidizing agents, by definition. Since Levan introduced the term, it has been used to describe the effects of many other substances, all of which are not necessarily polyploidogenic, although they may cause spindle suppression, chromosome scattering and other effects associated with colchicine. D'Amato (1948, 1949) has shown that both prophase and preprophase poisons may exhibit effects which resemble "C-mitosis." D'Amato and Avanzi (1949a) stated that true "C-mitotic" substances are those which do not interfere with the entry of interphases into prophase. Substances which cause prophase poisoning, prevent the entry of cells into mitosis (D'Amato and Avanzi, 1949 b). It has been recently shown by Hyypio (1954) that colchicine, in addition to causing polyploidy by suppressing spindle formation, also affects interphase nuclei at the stage called antephase (Bullough, 1952) and causes the appearance of scattered figures through the failure of an organized movement of prophase chromosomes to the

prometaphase clump. He pointed out that, although polyploidy and scattering of chromosomes may be found at the same time, their origin is quite different and that scattering leads to the formation of micro-nucleate cells and not polyploid cells.

MATERIALS AND METHODS

A. Experimental Procedure

The source of meristematic material for the experiments conducted was the root tips of germinated Pisum sativum var. Alaska seedlings. The seeds were furnished by the Ferry-Morse Seed Company from their 1952 and 1953 stocks. This strain has proven to give a fairly uniform mitotic index and "normal" mitoses, rendering it favorable for mitotic studies.

The seeds were placed in distilled water for a soaking period of six hours to hasten germination. The soaked seeds were then placed in paper toweling, moistened with distilled water for 60 - 72 hours for a germination and growth period. At the end of this time, seedlings with roots of approximately three to four centimeters in length were selected for treatment. This operation and all subsequent procedures were carried out at room temperature (about 24° C.).

The root tips were suspended in an aqueous solution for treatment. This was accomplished by the use of 250 ml. beakers as containers. Support was given by one-fourth inch mesh metal grids which had been coated with paraffin.

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The solvent used was a modified Hoagland solution as developed by Huskins and Stienitz (1948). The composition of one liter of full strength solution is:

.095 gm. $Ca(NO_3) \cdot 4H_2O$

.129 gm. NH_4NO_3

.180 gm. $MSO_4 \cdot 7H_2O$

.134 gm. KH_2PO_4

.007 gm. $K_2HPO_4 \cdot 3H_2O$

A quarter-strength nutrient solution was made up for use at the beginning of each run from stock solutions of the individual salts which were kept under refrigeration.

The terramycin used in these experiments was the HCl salt donated by the Charles Pfizer and Company, Incorporated, and bore the control number WBW527528. A solution was prepared at the beginning of each experiment. A solution of strong concentration was made and then diluted to the desired concentration in parts per million. Refrigerated stock solutions were not used due to the formation of a brown precipitate of terramycin which formed by hydrolysis of the salt, in solutions which were kept more than 48 hours.

At the time of treatment the 250 ml. beakers were filled with 220 ml. of the required solution. This included the concentration of terramycin - HCl to be tested dissolved in quarter-strength nutrient. Fifty to sixty seedlings could be suspended on the grids of each vessel for immersion.

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During the treatment period aeration and agitation was supplied by air bubbled through glass tubes with small openings. The air supply was from the laboratory outlet, and was passed through a charcoal filter to remove any foreign material.

Four root-tips were collected at the beginning of each experiment and were used as zero hour controls. All subsequent cuttings included four root-tips from each test vessel and control according to times required by the mode of treatment.

The root-tips were placed in three parts 100 percent ethanol to one part glacial acetic acid after each collection for fixation under vacuum for one to two hours. A hydrolysis period of nine minutes in 1N HCl at 60°C followed fixation. The Feulgen reaction was used for staining and slides were made by the squash technique. The slides were then microscopically checked before being immersed in 95 percent ethanol for 12 to 24 hours for dehydration. A small amount of fast green had been added to the ethanol as a counterstain. The slides were made permanent with Diaphane.

B. Treatment

The experiments were planned with the goal of reaching a dosage threshold at which effects and aberrations could be studied. The first trial runs were set up using continuous

treatments in aqueous media. Fifty ppm., 100 ppm., and 200 ppm. concentration of terramycin · HCl was used with collections at zero, one, two, four, six, and eight hours. At the end of treatment, the roots were thoroughly washed and placed in moist paper toweling for recovery.

Upon examination of the slides, it was ascertained that these dosages soon reduced the mitotic index to a point where it was difficult to study aberrations. Therefore, another run using the lower concentrations of 25 ppm., and 10 ppm. was set up according to the same schedule as before. At these concentrations a decrease in mitotic index was not realized, but there was a scarcity of affected figures.

To try to overcome these difficulties, it was decided to use short term treatments, followed by a recovery period in quarter-strength nutrient. One hundred ppm. and 200 ppm. concentrations were used, with treatment for 15 minutes. The roots were then thoroughly washed and transferred to quarter-strength nutrient. Collections were made at zero hour (control), 15 minutes treatment; one-quarter, one-half, one, two, three, four, five and six hours recovery. Following this method of treatment, there were few aberrations and recovery was realized within a few hours.

It was then decided to use the higher concentrations of 600 and 800 ppm. and increase the treatment period to 30 minutes. Collections were made after zero, fifteen and

thirty minutes treatment. The roots were thoroughly washed and transferred to quarter-strength nutrient for recovery, with collections at one-quarter, one-half, one, two, three, four, five, six, seven and eight hours.

C. Cytological Examination

The slides were microscopically examined using a 90 X oil immersion objective and 12.5 X oculars. Lighting was supplied by a ribbon filament lamp, using a type B green filter and a type E orange filter between the light and microscope mirror.

Quantitative analysis were made by examinations 50 units apart on the horizontal scale. At least three planes were checked. One thousand cells were counted to determine the mitotic index. All mitotic cells were classified according to phase and type of aberration until a total of one hundred was reached. Any abnormalities seen in resting nuclei were also noted.

Data taken from examination of the slides, was kept on score sheets. These were used as a basis for a quantitative analysis of mitotic effects.

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OBSERVATIONS

A. Classification of Aberrations

Prophase

To facilitate analysis prophase was divided into four categories: early, mid, and late prophase; and pro-metaphase. A plate of photomicrographs of the main types of abnormalities is included at the end of this thesis. (Plate III)

Early prophase. No abnormalities were noted in this category.

Mid-prophase. Reversions were the main type of abnormality. These are characterized by a relaxation of the coils and a general diffuse appearance. A few spread configurations were also seen, with the chromosome threads loosely arranged within the nuclear membrane.

Late prophase. The greatest number of aberrant cells were found in this stage. They had chromosomes of varying degrees of contraction and reversions. Contracted figures were classified as "slightly contracted," "moderately contracted," and "severely contracted." The slightly contracted figures are recognized by a thickening of the chromosomes; the severely contracted figures are of metaphase length and scattered

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around the periphery of the nuclear membrane; with the moderately contracted figures in an intermediate category. Reversions were observed in normal late prophases and all types of contracted figures.

Pro-metaphase. No abnormal figures were observed in this stage.

Post-Prophase

Post-prophase was classified according to the traditional metaphase, anaphase and telophase.

Metaphase. Laggards, slightly scattered figures, severely scattered figures and reversions were found. Laggards are chromosomes which sit apart from the rest of the chromosomes. In the slightly scattered figures, the chromosomes are grouped towards the center of the cell, but the kinetochores do not line up on a plate. In severely scattered figures the chromosomes are widely spread throughout the cell. These are distinguished from the severely contracted figures in late prophase by a separation of the chromatids except at the kinetochore region. A few reversions of the severely scattered figures were observed.

Anaphase. Abnormalities were classified as laggards, bridges, spread figures, scattered figures and reversions. Bridges are strands of chromatic material between the

separating chromosome groups. These may be due to stickiness of the matrix or to entanglement of chromosomes with trabants. Spread figures are typified by two groups of chromosomes, in which the chromosomes are somewhat shortened and without a close orientation of kinetochores towards the poles. In the scattered figures, the chromosomes form only one group, with sister chromatids side by side. Reversions of scattered figures were occasionally observed.

Telophase. Spread figures and bridges were the types of irregularities noted. The morphology is the same as in anaphase, with the exception of a telomorphic appearance.

Interphase

Pycnotic and binucleate cells were observed, but a quantitative analysis of them was not made.

B. Continuous Treatment

General Observations

A deviation from the normal condition was observed in the development of a root system in the recovery material (Plate I). After a recovery period of six days the material which had been treated with 200 ppm. terramycin • HCl had no side-roots and was shorter in length than the control. Those which had been in 100 ppm. had an occasional very short

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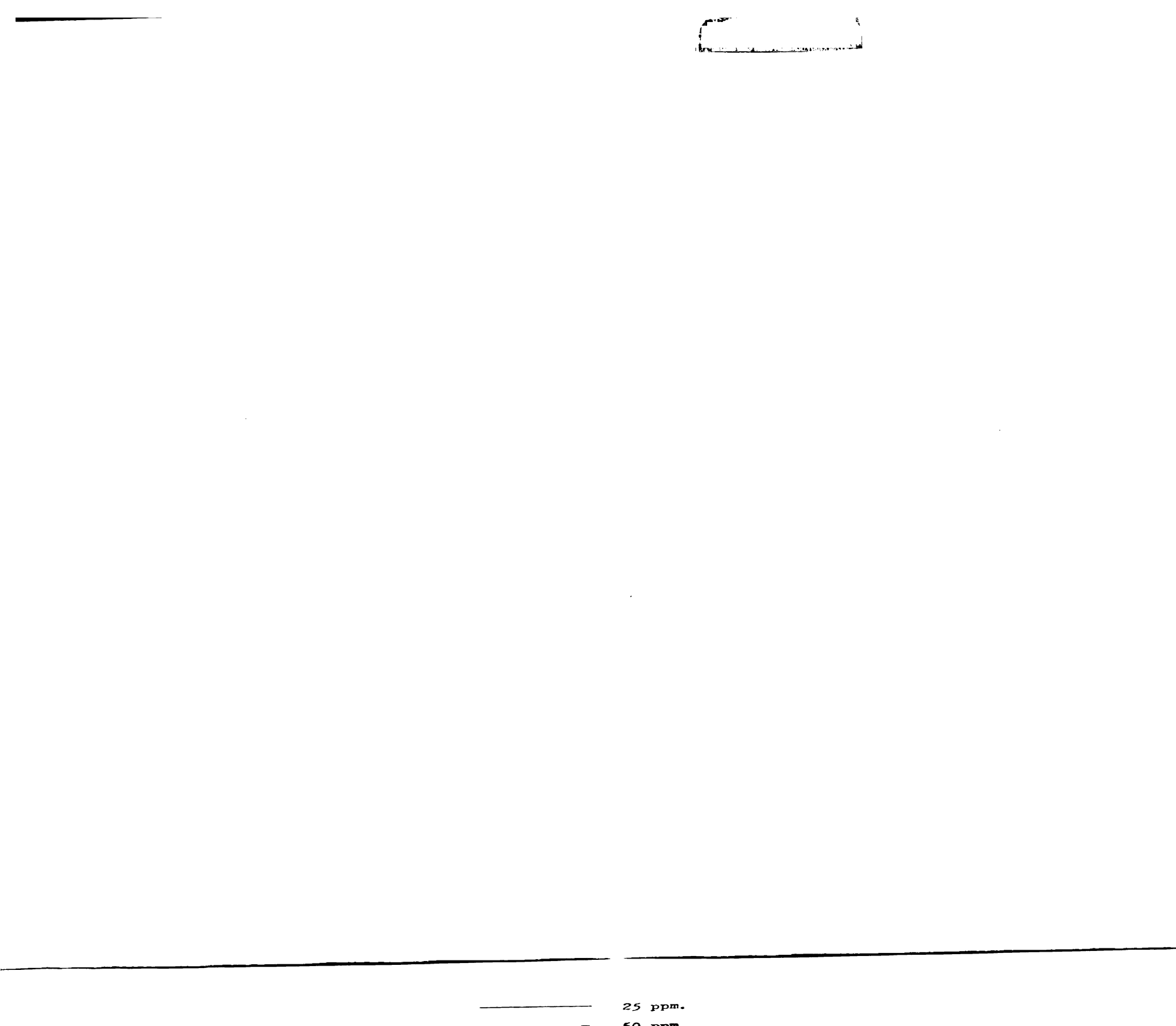
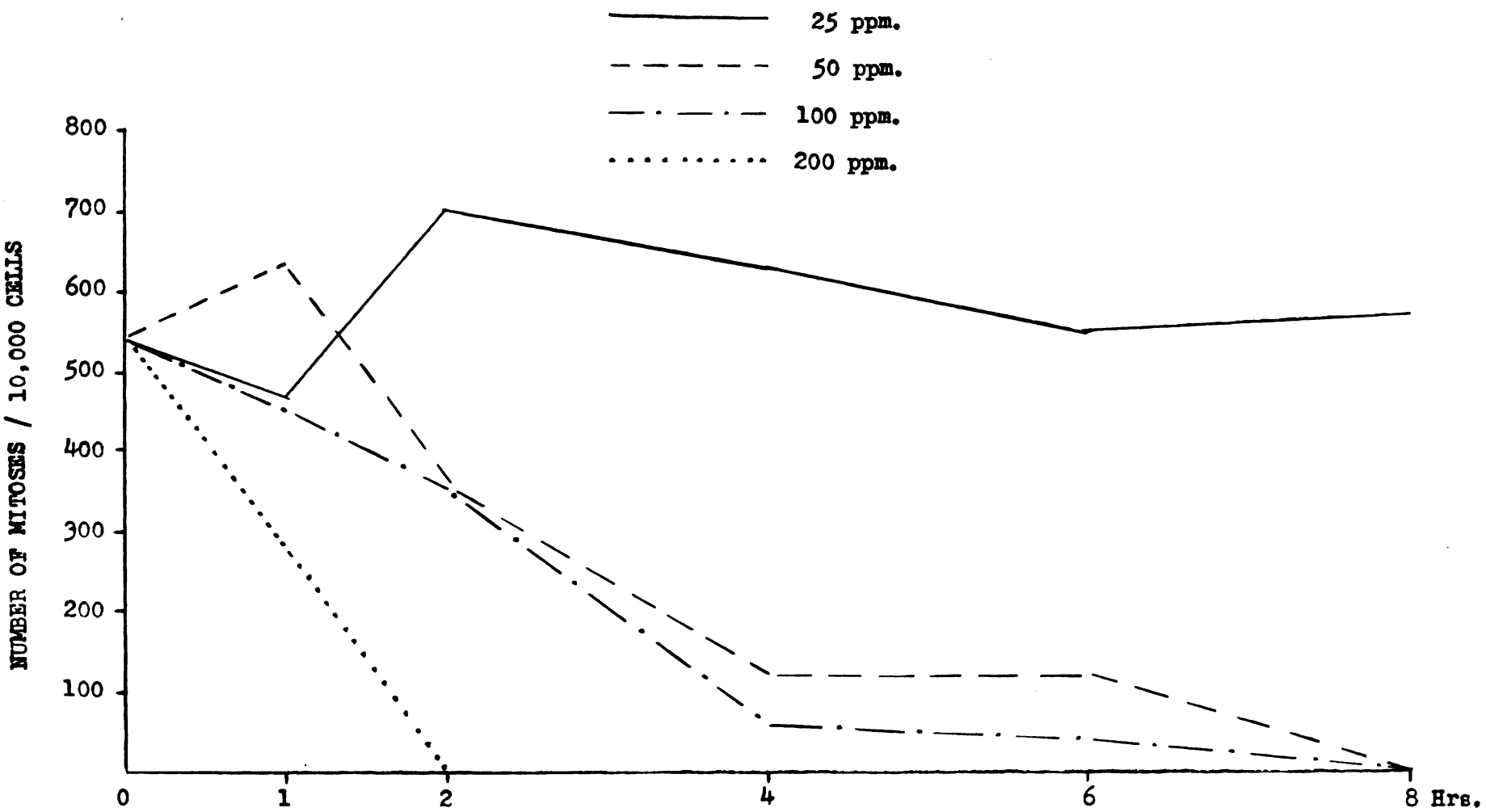
side-root above the region of immersion, and none in the new growth region. They, also, were shorter than the controls.

After a ten day recovery period, the 50 ppm. treated roots showed only an occasional small side-root above the immersion region and a few short side-roots from the new growth. Those from 25 ppm. treatment, had only an occasional side-root above the immersion region and had several side-roots in the region of new growth. The main roots were shorter than those of the controls. . The 10 ppm. material deviated from the control only in a lack of side roots in the immersion region.

Analysis of Mitotic Index

In text Figure 1, a comparision of the mitotic indexes for the various concentrations used is shown. Ten ppm. concentration of the antibiotic was not graphed, as it follows the same trend as 25 ppm. concentration. At 25 ppm., no significant increase or decrease was realized in the mitotic index. The mitotic index of material treated with 50 ppm. showed a very slight increase at the first hour and then decreased gradually to zero at eight hours of treatment. At 100 ppm. treatment, the mitotic index dropped steadily for the first four hours and then tapered off to zero at eight hours. The mitotic index of material treated with 200 ppm., dropped abruptly to zero within two hours of treatment.


25 ppm.



Text Figure 1. Variation of Mitotic Index

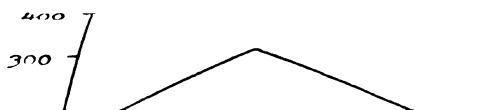
Continuous Treatment

Analysis of Abnormalities as Compared to the Total Number of Mitotic Cells

In this analysis of continuous treatment the concentrations of 50 ppm. and 100 ppm. were used. The concentrations of 25 ppm. and 10 ppm. did not show enough aberrations and in 200 ppm. there were insufficient data.

The absolute number of total prophase figures in 50 ppm. treated material, decreased with duration of treatment as is shown in text Figure 2a. The proportion of these figures which was abnormal increased with treatment. At the end of two hours, no pro-metaphases were observed. Early prophase dissappeared rapidly and were no longer in evidence by the end of four hours. Also, the total number of mid-prophases decreased gradually during treatment. This stage showed reversions as early as one hour, with the proportion of reversions to normals increasing gradually during treatment, until at the end of six hours only a few normal figures were left.

The proportion of late-prophases to total-prophases increased gradually during treatment, as is seen by a comparison of text Figure 2a with text Figure 2b. The first hour of treatment showed an increase in the number of normal late-prophases, with a few contracted figures and reversions. At two hours the number of contracted figures, the degree of contraction, and the number of reversions had increased. At four hours severe contraction was in evidence and reversions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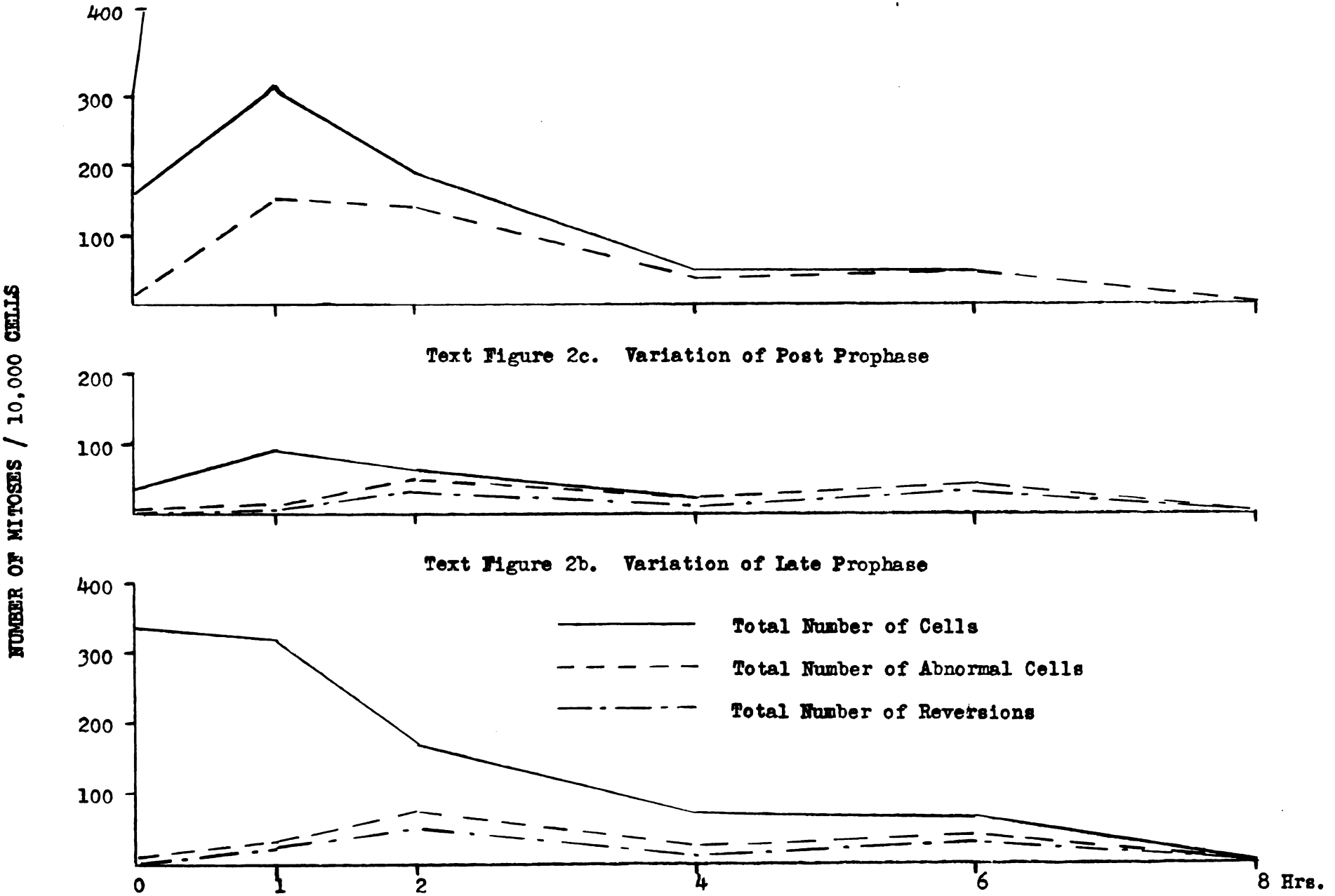


Text Figure 2c. Variation of Post Prophase

Text Figure 2b. Variation of Late Prophase

Text Figure 2a. Variation of Total Prophase

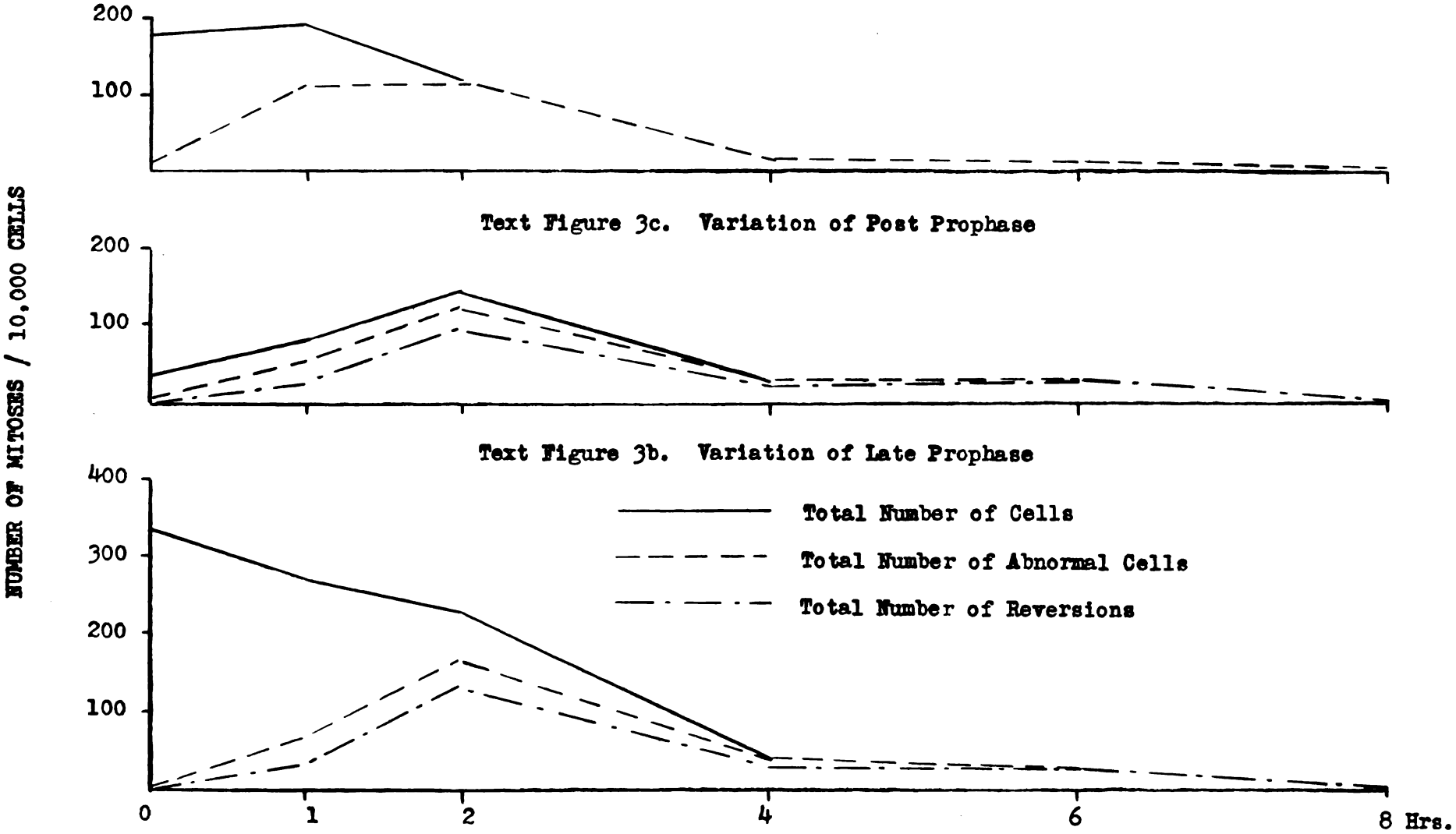
50 ppm.; Continuous Treatment

were mainly of contracted figures. By six hours no normal figures were found.

Text Figure 2c shows the analysis for post-prophase. There was an increase in absolute number the first hour, followed by a gradual decline to zero by the end of eight hours. As treatment continued, the proportion of abnormals to normals increased. These consisted mainly of slightly scattered metaphase figures in the first hour of treatment, which were replaced with severely scattered figures by four hours. Abnormalities in anaphase and telophase were mainly of spread figures, with occasional bridges in both normal and spread figures. A few scattered figures in anaphase, and reversions thereof, were also noted. In all three phases laggards were found during the whole course of treatment.

The picture in 100 ppm. treated material was generally the same as in 50 ppm, but the effect was more pronounced as is shown in text Figure 3. Again the absolute number of figures decreased, but more rapidly than in 50 ppm.

Also the occurrance and proportion of abnormalities was greater. All figures were abnormal after four hours of treatment. Both early-prophase and pro-metaphase disappeared within two hours. Mid-prophase had virtually disappeared by the end of four hours of treatment, with the majority being of the reverting type before that time.



Text Figure 3c. Variation of Post Prophase

Text Figure 3b. Variation of Late Prophase

Text Figure 3c. Variation of Total Prophase

100 ppm.; Continuous Treatment

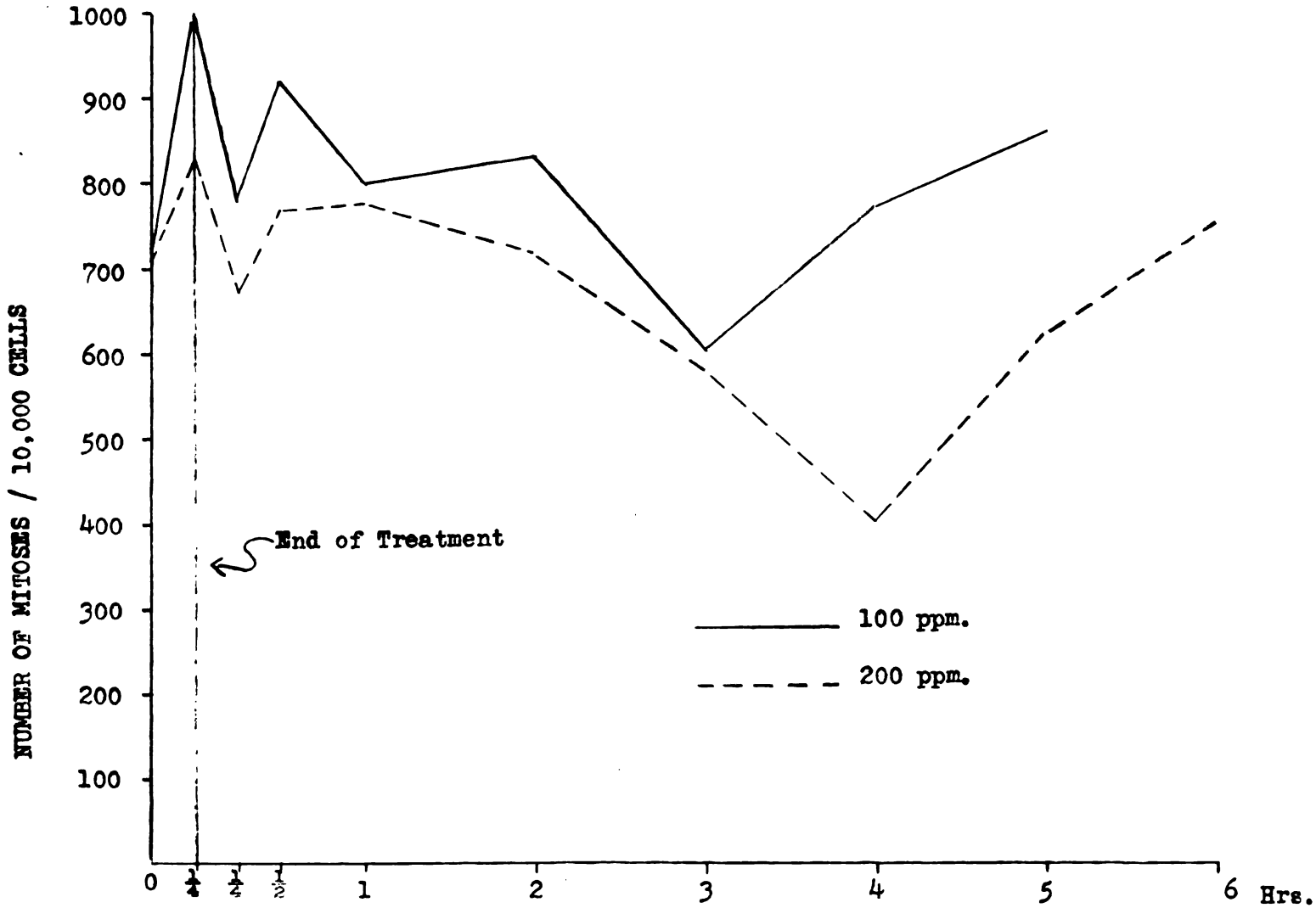
The proportion of late-prophase to total-prophase was greater than in 50 ppm. Contracted figures and moderately contracted figures were in evidence by the first hour of treatment, as well as several reversions of normal figures. By four hours, nearly all figures were ~~of~~ reversions of moderately and severely contracted types; and by six hours only reversions of these contracted figures could be found.

Post-prophase also showed the same general trend, but to a greater degree. Severely scattered metaphase figures were found earlier and in greater proportions. These severely scattered figures and reversions of them were the only type found in the later hours. Otherwise, the picture is the same as in 50 ppm. treatment.

C. Fifteen Minute Short Time Treatment and Recovery

Analysis of Mitotic Index

A comparison of the mitotic indexes of material treated with 100 ppm. and 200 ppm. is shown in text Figure 4. The mitotic index of material treated with 100 ppm. shows a sharp increase at the end of 15 minutes of treatment. One-quarter to two hours recovery showed fluctuation. At three hours recovery the mitotic index dropped, and then again went up at four and five hours recovery. The 200 ppm. treated material did not show as sharp an increase at the end of 15



Text Figure 4. Variation of Mitotic Index.

15 Minutes Treatment and Recovery Period

minutes treatment. There was a drop in the mitotic index at one-quarter hour recovery and a small increase at one-half hour recovery with a steady decrease until four hours of recovery time was reached. The mitotic index then increased until it reached the normal state by six hours.

Analysis of Abnormalities as Compared to Total Number of Mitotic Cells

The absolute number of total-prophase figures in material treated with 100 ppm. for 15 minutes, showed a fluctuation from 15 minutes treatment to one hour recovery. The total number of figures then decreased gradually until a low at three hours was reached, followed by an increase, as is shown in text Figure 5a.

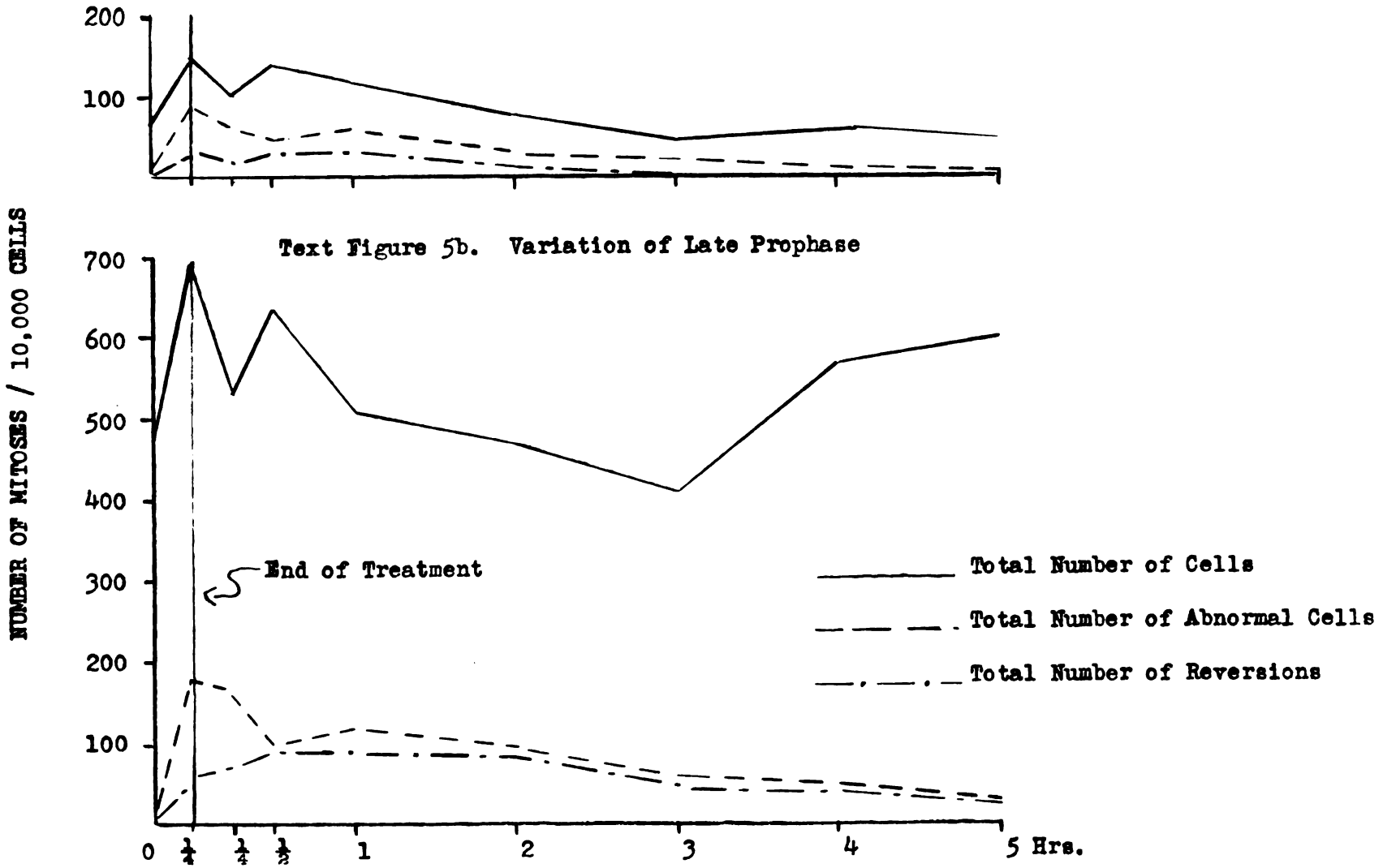
The proportion of early-prophase was never very low, but reached the lowest frequency at three hours, and the highest at four and five hours. Pro-metaphase varied very little during the course of treatment and recovery. Mid-prophase also showed little proportional variation, with the lowest proportions at four and five hours. Reverting figures appeared in this stage, with the highest frequency at the end of treatment and one-quarter hour recovery, followed by a gradual decline to the end of the recovery period.

There was no marked increase in the proportion of late-prophase to total-prophase, as is shown by a comparison of

text Figure 5b with text Figure 5a. The majority of abnormalities were of the slightly and moderately contracted types. Reversions were found in a small proportion from 15 minutes treatment to three hours recovery and were of normal and contracted figures.

Post-prophase analysis is shown in text Figure 6. The total number of figures fluctuated during 15 minutes treatment and one-quarter hour recovery, then climbed gradually to a high at two hours of recovery. There was a drop at three hours recovery followed by a gradual rise to five hours of recovery. The proportion of abnormal cells was low, reaching the highest proportion at two and three hours of recovery. Abnormalities in metaphase were of slightly scattered figures at two and three hours recovery. Spread figures were the main type of abnormality observed in anaphase and telophase. Some laggards were observed in all post-prophase stages.

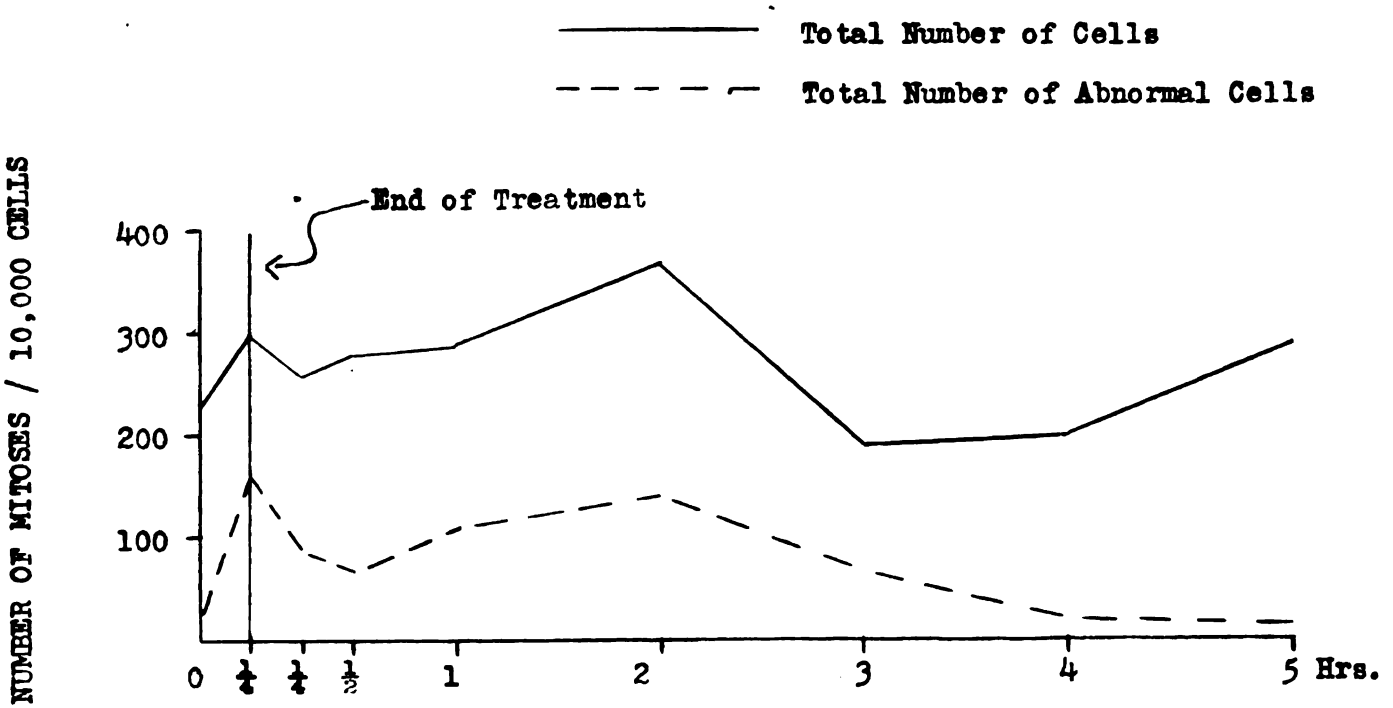
The results obtained from treatment with 200 ppm. for 15 minutes, followed the same general trend as 100 ppm. treatment, as is shown in text Figures 7 and 8. With 200 ppm. the initial increase of total prophase at the end of treatment was not as great as in 100 ppm. A decrease at one-quarter hour recovery was followed by an increase at one-half hour recovery, and then a gradual decline to a low



Text Figure 5b. Variation of Late Prophase

Text Figure 5a. Variation of Total Prophase

100 ppm.; 15 Minutes Treatment and Recovery Period



Text Figure 6. Variation of Post Prophase

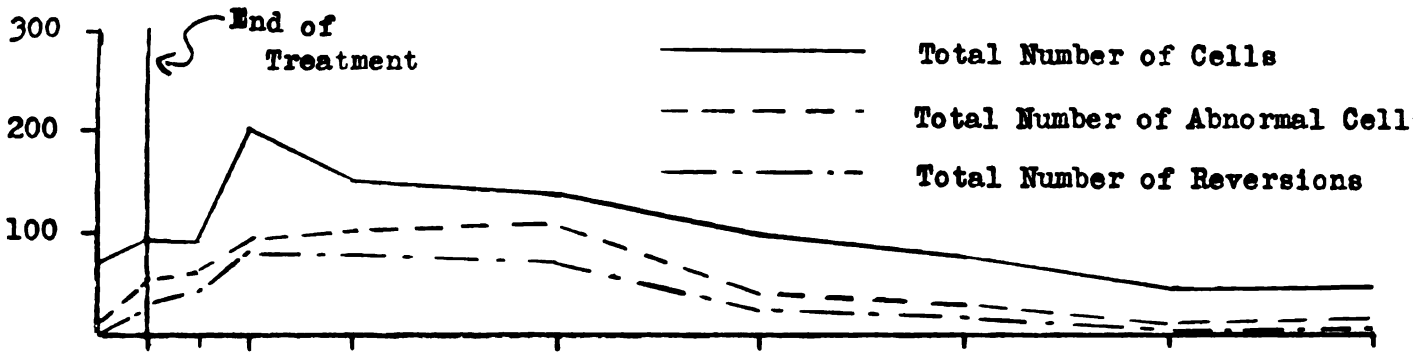
100 ppm.; 15 Minutes Treatment and Recovery Period

at four hours of recovery. At five and six hours recovery there was an increase in the total number of prophase stages.

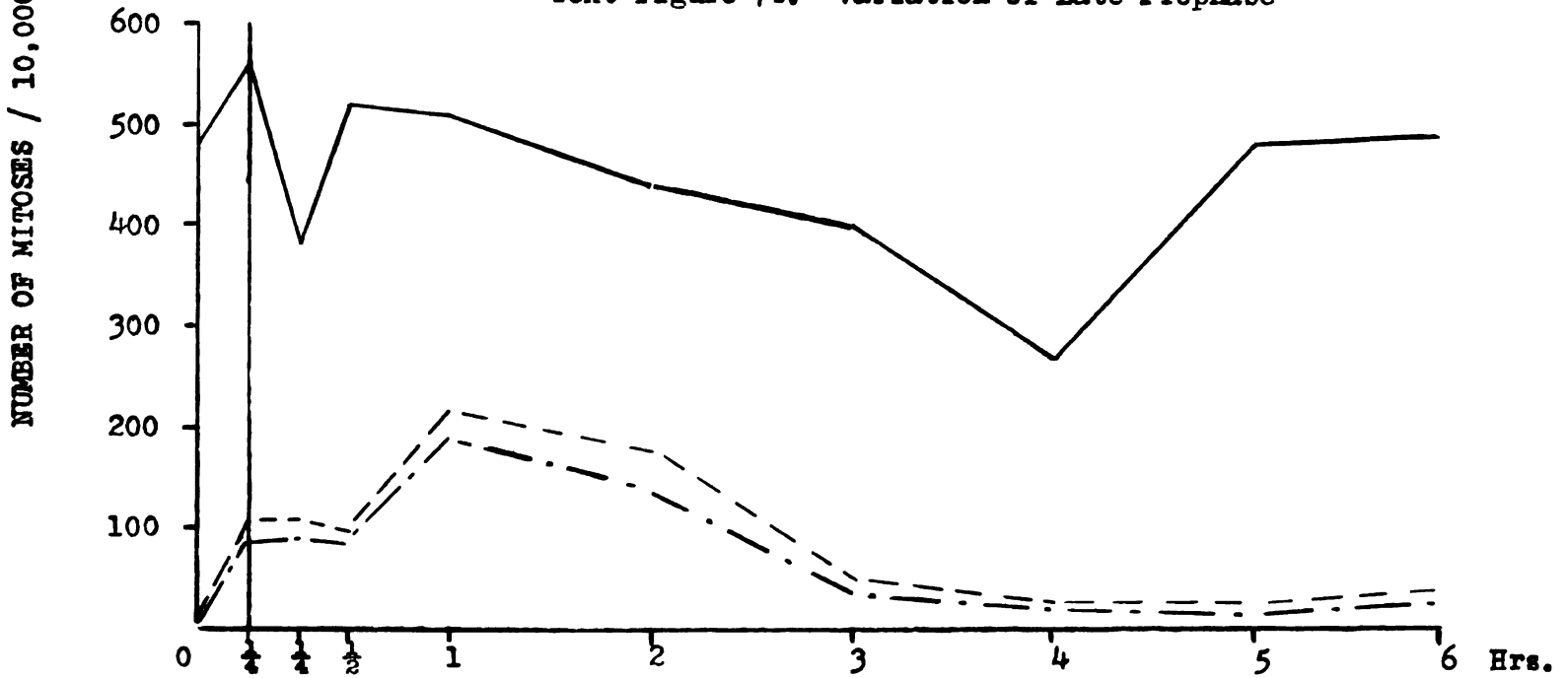
As in 100 ppm., early-prophase stages were always found; however, the decrease in number was more pronounced with the lowest point at four hours of recovery. Pro-metaphase stages were found at all points, except three hours recovery. Reverting figures were noted in this stage with the highest proportions at one and two hours of recovery, followed by a gradual decline to six hours recovery.

Late prophase showed a greater decrease in proportion to total-prophase than in 100 ppm. There was a sharp increase in this stage at one-half hour recovery and then a gradual decline to six hours recovery. More contracted figures and more severe contraction was seen than in 100 ppm., and was most evident at two and three hours of recovery. Reversions of normal and contracted figures were found with the highest proportion at one to three hours recovery.

A graph of post-prophase analysis for 200 ppm. is presented in text Figure 8. Again, there is a similarity to 100 ppm. treatment. The total number of figures varied only slightly to two hours recovery, after which it dropped to a low point by four hours, followed by a gradual increase to six hours. As in 100 ppm., there was always a good proportion of normal figures present. The highest

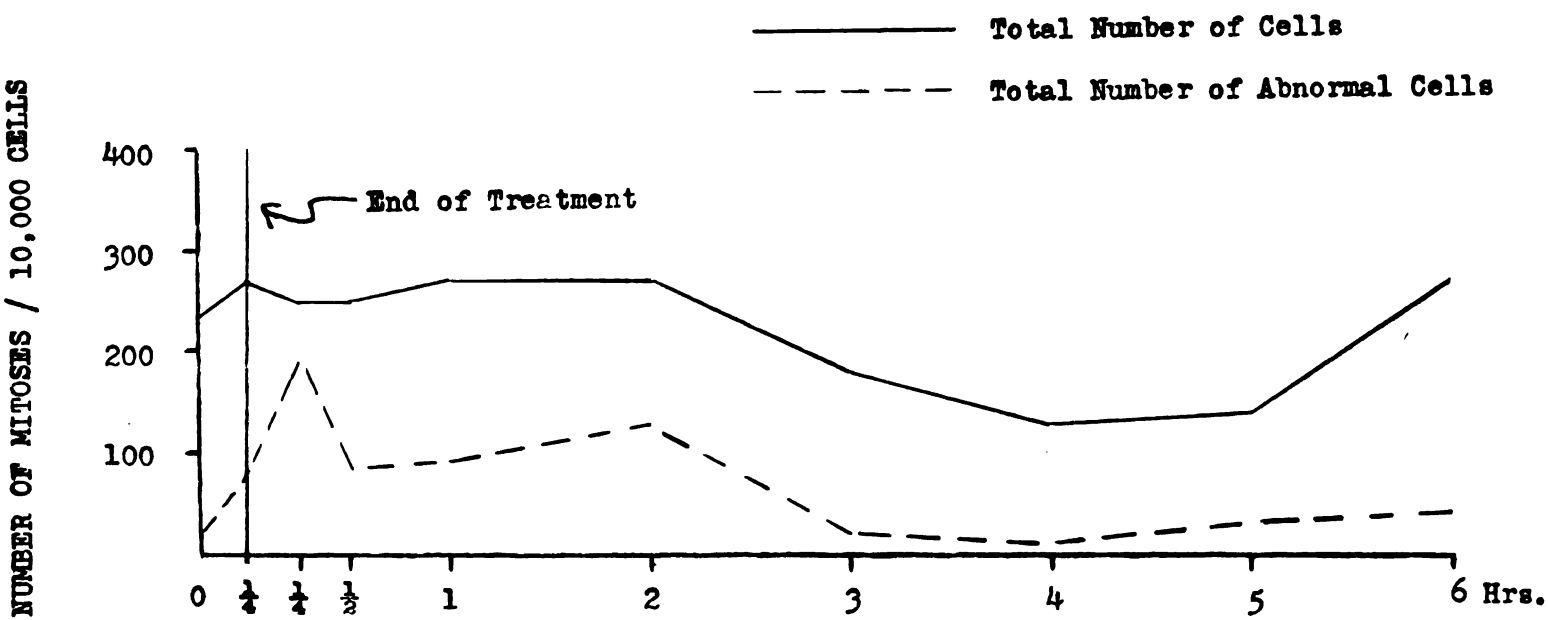


Text Figure 7b. Variation of Late Prophase



Text Figure 7a. Variation of Total Prophase

200 ppm.; 15 Minutes Treatment and Recovery Period



Text Figure 8. Variation of Post Prophase

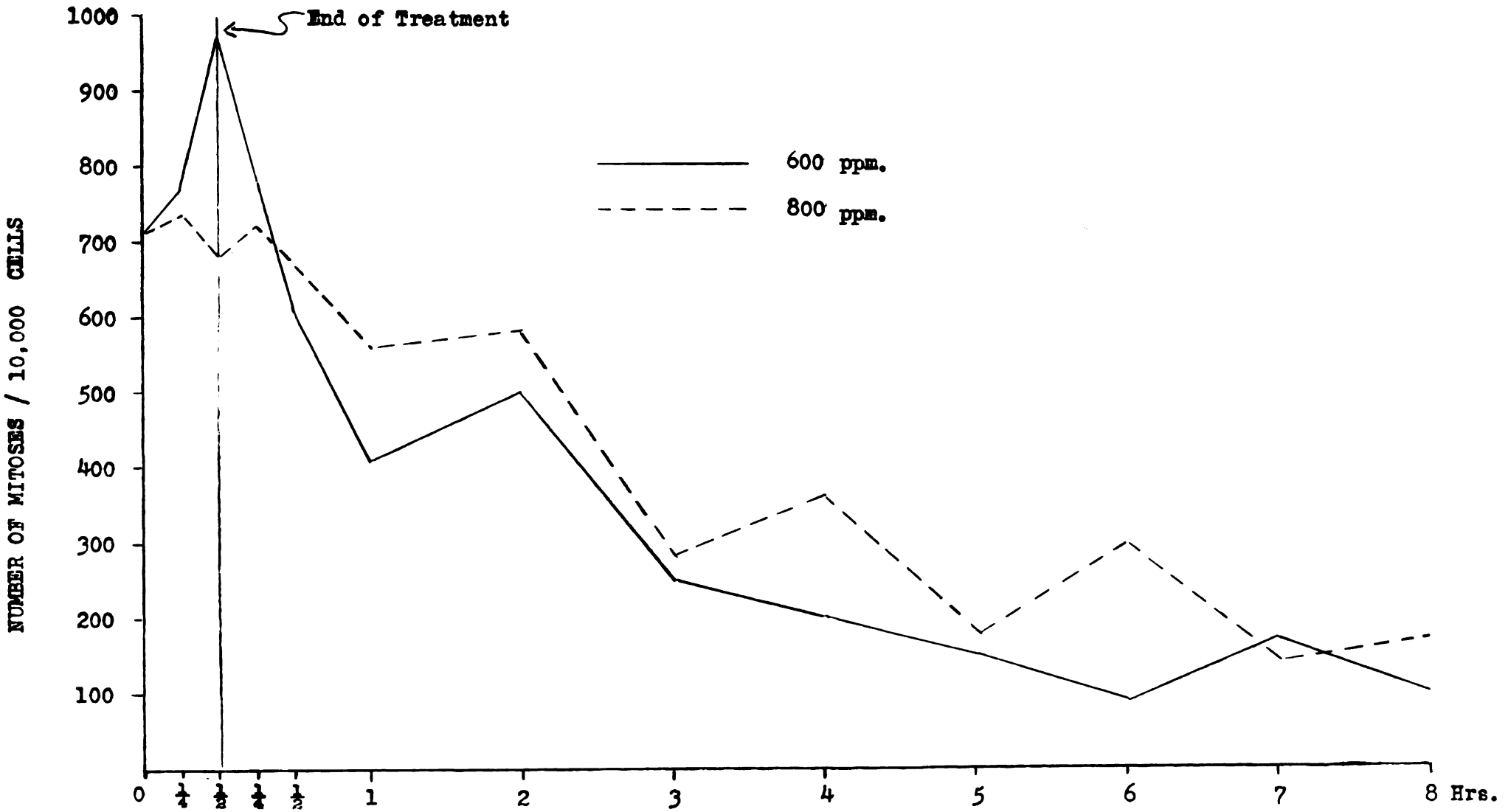
200 ppm.; 15 Minutes Treatment and Recovery Period

proportion of abnormalities was realized at one-quarter hour recovery with a gradual decline in frequency thereafter. Abnormal figures were the same as in 100 ppm. treatment, with the exception of the appearance of more severely scattered metaphase figures at one and two hours recovery, and their maintenance in subsequent recovery hours.

D. Thirty Minute Short Time Treatment and Recovery

Analysis of Mitotic Index

A comparison of the mitotic indexes of roots treated with 600 ppm. and 800 ppm. concentration for 30 minutes, followed by a recovery period of eight hours is given in text Figure 9. Six hundred ppm. treatment showed an increase at the end of the 30 minute treatment period and a decline to one hour recovery. At two hours of recovery there was a slight increase, followed by a gradual decrease to a low at six hours recovery. At seven hours recovery there was an increase, again followed by a slight decrease at eight hours of recovery. The 800 ppm. treatment did not show an increase at the end of treatment, and exhibited a gradual decrease, with hourly fluctuation until a low was reached at seven and eight hours recovery.



Text Figure 9. Variation of Mitotic Index.

30 Minutes Treatment and Recovery Period

Analysis of Abnormalities as Compared to the Total Number of Mitotic Cells

A graphic analysis of total-prophase figures for 600 ppm. treatment is presented in text Figure 10a. The number of figures increased to a high at the end of the treatment period, followed by a sharp drop to one hour recovery. There was an increase at two hours recovery followed by a gradual decline to six hours, another small increase at seven hours and a decline at eight hours recovery. As the number of total figures dropped the proportion of abnormal figures increased.

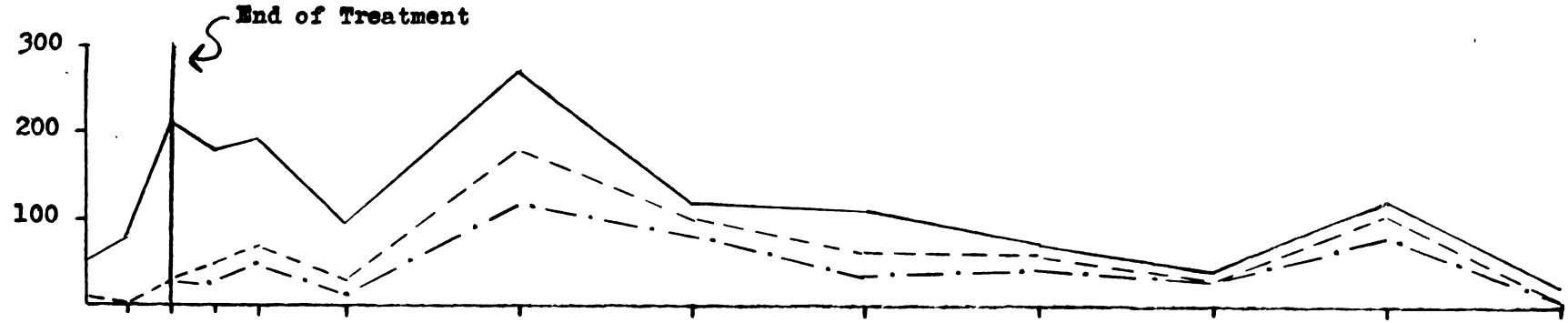
Early prophases were always noted; however, the frequency showed a sharp drop by one hour recovery and was very low until six hours recovery, at which time there was a gradual increase. Pro-metaphases disappeared at two hours recovery and did not reappear until seven hours recovery time. The frequency of mid-prophases realized a sharp drop within one hour of recovery and was maintained only in small numbers thereafter. The proportion of reversions, in this stage, reached a high point at one-half hour recovery and remained fairly stable until six hours recovery when a slight increase was realized.

There was a marked increase in the proportion of late-prophase to total prophase during the duration of the treatment, as is shown by a comparison of text Figure 10a with text

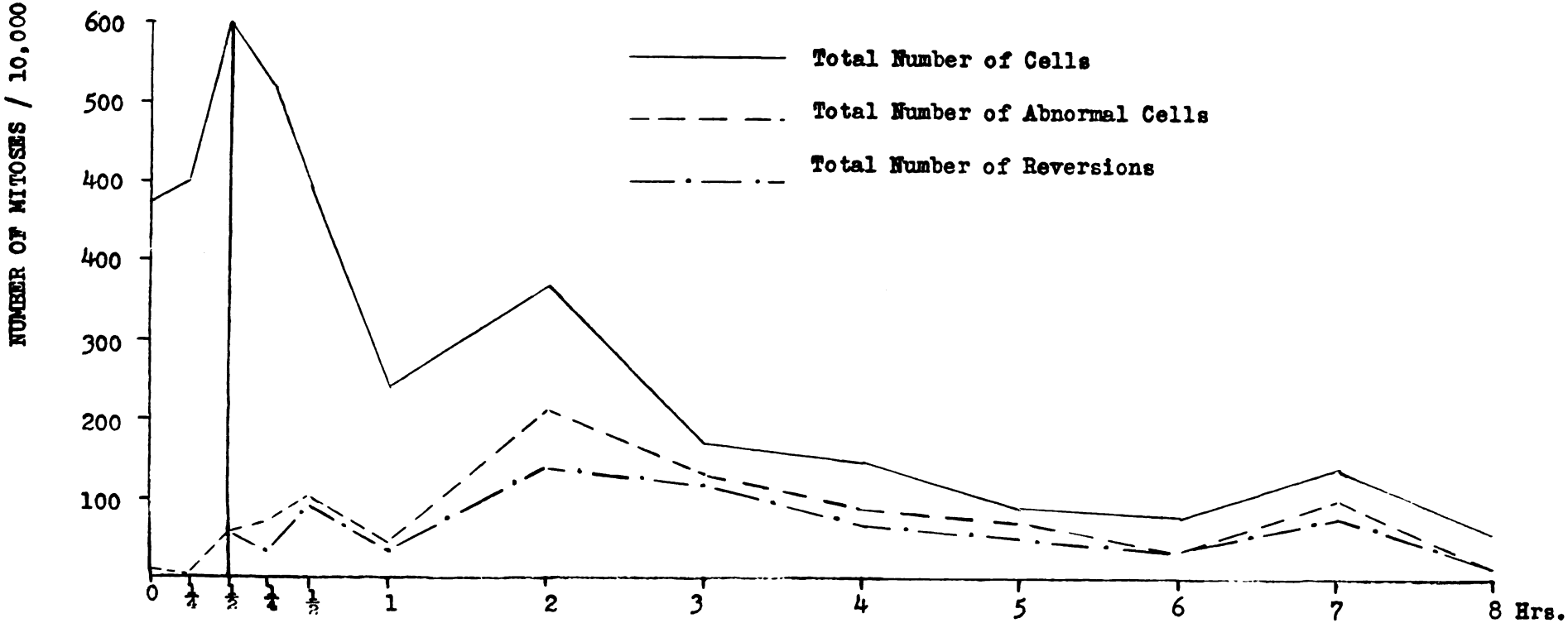
text Figure 10b. By one hour recovery the proportion of late-prophase had showed a sharp increase and continued to rise until five hours recovery, when it started to decrease slowly. Abnormalities were of contracted figures and reversions, with the most severely contracted figures becoming more predominant with duration of time.

Text Figure 11 shows the analysis of post-prophase for 600 ppm. treatment. There was a gradual decrease of total figures to four hours recovery, after which the total number remained fairly constant to the end of the recovery period. As in prophase, the proportion of abnormal figures increased with the decrease in number. Abnormalities in metaphase consisted of slightly scattered figures until one hour recovery, after which they were replaced gradually by severely scattered figures. A few reversions were observed in the later recovery hours. Anaphase irregularities were of spread figures and scattered figures. Bridges were evident and were due to matrix stickiness or entangled trabants. Spread figures and bridges were found in telophase. As in the other treatments, laggards were seen in all post-prophase stages.

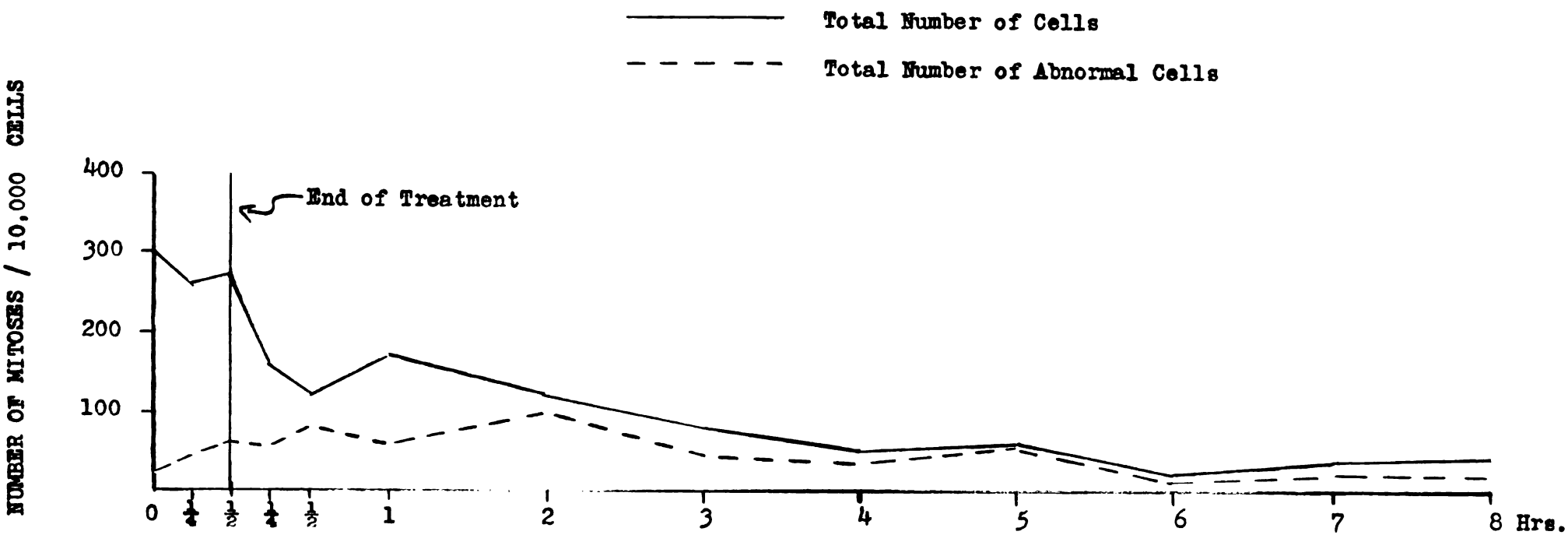
Total prophase analysis for 800 ppm. follows the same trend as 600 ppm. treatment, as is shown in text Figure 12. There was a gradual fluctuating decrease in the total number of prophases, reaching a low at seven and eight hours recovery.



Text Figure 10b. Variation of Late Prophase



Text Figure 10a. Variation of Total Prophase
600 ppm.; 30 Minutes Treatment and Recovery Period.



Text Figure 11. Variation of Post Prophase

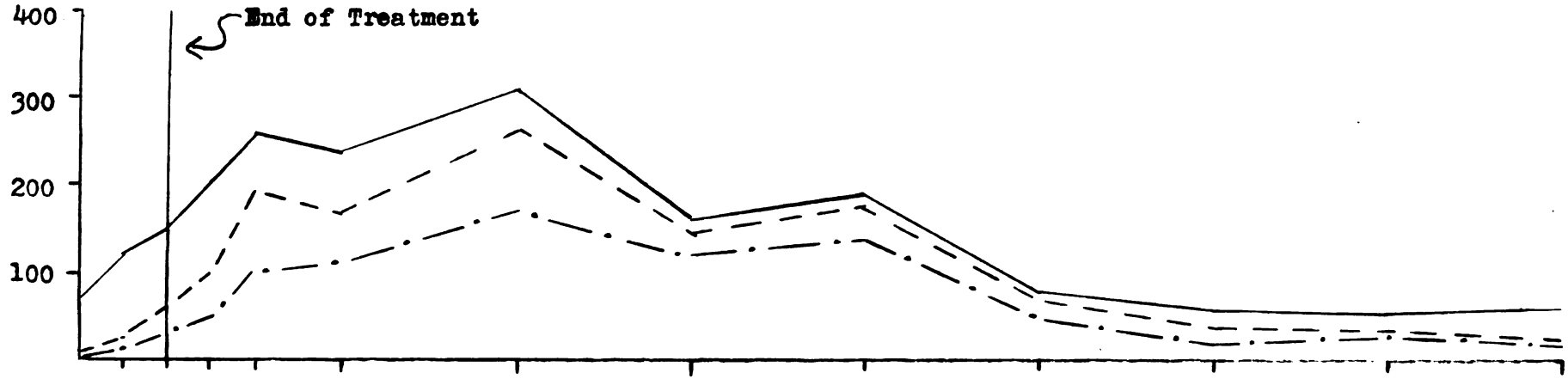
600 ppm.; 30 Minutes Treatment and Recovery Period

The frequency of early-prophase figures dropped quickly at the end of treatment and early hours of recovery, reaching nearly zero at four and five hours recovery. They also followed the same trend as was seen in 600 ppm. treatment. From one hour recovery to five hours recovery, late-prophase was the main class of prophase figure. After five hours the proportion of late prophase began to decrease gradually. Abnormalities were of the same type and followed the same trend as was noted in 600 ppm. treatment.

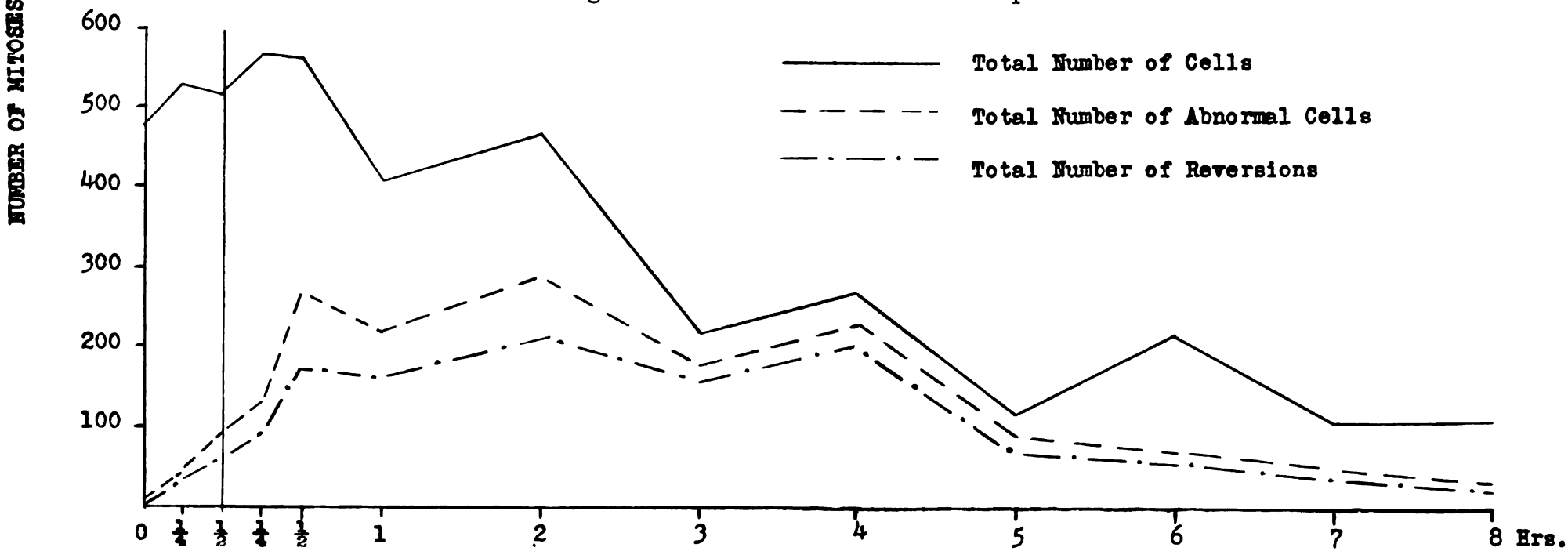
Post-prophase effects for 800 ppm. are presented in text Figure 13. Absolute numbers, proportion of abnormalities, and types of abnormalities followed the same general pattern as was seen in 600 ppm. treatment.

E. Summary of Observations

1. Continuous treatments reduced the number of side roots in recovery material; the degree depending on the concentration.
2. The mitotic index showed a decrease in all treatments. depending on the concentration used and length of treatment.
3. The absolute numbers of total prophase figures decreased in all cases, varying with concentration and length of treatment used. The greatest proportional decrease was

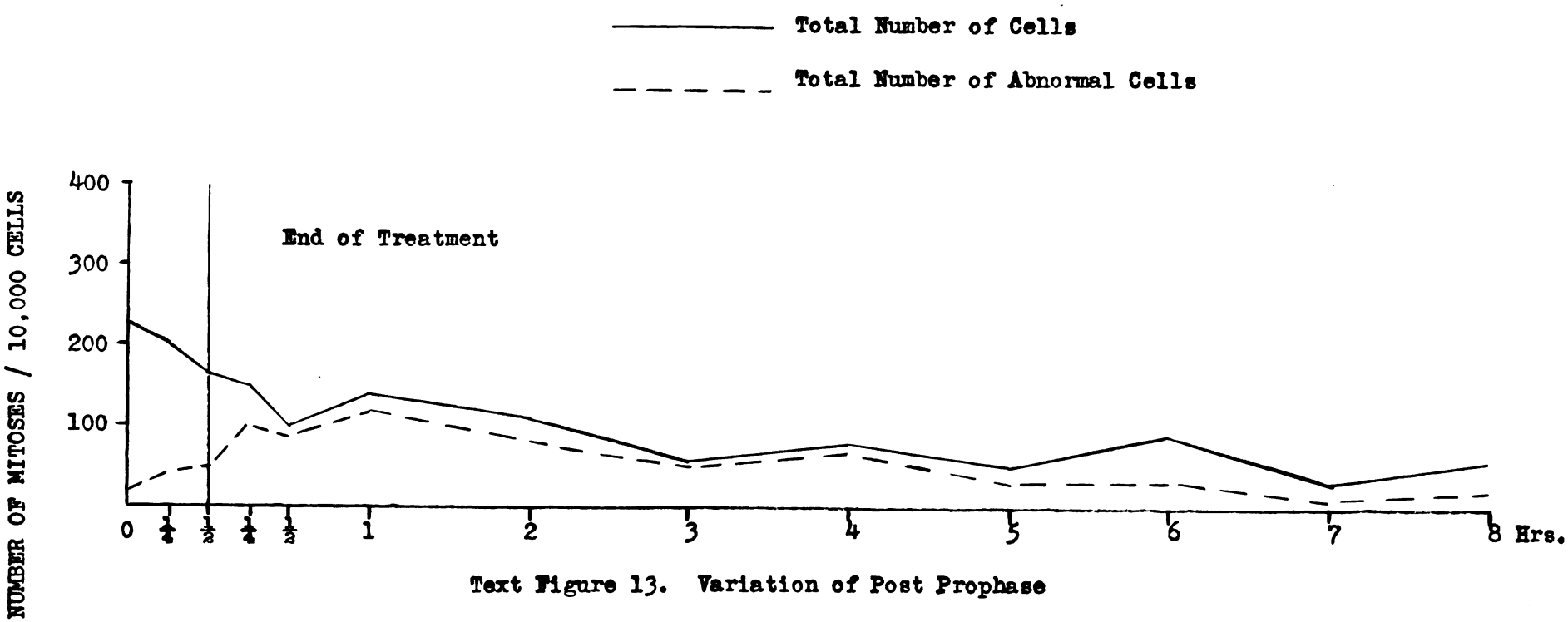


Text Figure 12b. Variation of Late Prophase



Text Figure 12a. Variation of Total Prophase

800 ppm.; 30 Minutes Treatment and Recovery Period



Text Figure 13. Variation of Post Prophase

800 ppm.; 30 Minutes Treatment and Recovery Period

in early prophase and pro-metaphase. Mid-prophase also showed a proportional decrease in figures and a rise in reversions.

4. Late-prophase figures showed a proportional increase to total-prophase figures, with duration of treatment or recovery. Abnormalities were of contracted figures and reversions, with degree and proportion of contraction and the proportion of reversions increasing with time. A correlation of abnormalities with concentration and length of treatment was observed.

5. The absolute numbers of post-prophase figures decreased in all treatments according to concentration and length of treatment used. The proportion of abnormal figures increased as the total number of figures decreased. Abnormalities in metaphase consisted of scattered figures, with degree and proportion dependent upon time and the mode of treatment. Anaphase abnormalities were mainly of scattered and spread figures. The abnormalities observed in telophase were mainly spread figures.

DISCUSSION

Normal Cycle

In order to understand what has gone wrong in an effected mitotic cycle, it is first necessary to know the normal mitotic cycle. For the sake of convenience, the normal process of mitosis has been classified into several stages or phases. However, it must always be kept in mind, that these terms are nothing but names given to certain stages in a constantly moving and changing system, which results in the formation of two nuclei and cells from one nucleus and cell.

During the process of mitosis, the chromosomes go through observable morphological changes and movements. Interphase is diffuse and granular in appearance. At prophase, definite spiralled threads can be seen. At the beginning of prophase the kinetochores are arranged around the old telophase pole and form a hollow sphere. As this stage proceeds, the chromosomes shorten and become thicker. Simultaneously, the kinetochores move towards the equator of the nucleus. Usually before they reach this point, however, the chromosomes move to the center of the cell. They are then in the usual pro-metaphase arrangement of an irregularly shaped

clump. This centerward movement appears to be associated with disruption of the nuclear membrane.

While in pro-metaphase, more contraction and unwinding of relational coils is realized. It is possible that the spindle, which was probably formed sometime during prophase, may become functional and have some influence along with the kinetochores on the movement of the chromosomes to the periphery of the equatorial plate. This movement results in the formation of the metaphase plate. While at the metaphase plate, the kinetochores cleave. This is followed by a mutual repulsion of sister kinetochores. The forces of the spindle then come into operation and the resulting configuration is called anaphase. The sister chromatids move apart, with the kinetochore leading and the arms trailing behind. Telophase is the name given to the stage when the chromosomes reach the poles and begin the morphological transformation towards interphase. During late anaphase and in telophase, cytokinesis occurs and the nuclei are separated into two different cells. This involves, among other things, the loss of matrix and some relaxing of the chromonema coils.

This account of mitosis is concerned only with those things which can be observed. Schrader (1953) has reviewed many of the theories of mitotic movements. Most of them are highly theoretical and can not even be proved with present

techniques. Therefore, not much would be gained from a discussion of them in a study of this sort. The basic theory, followed in this research, was the one proposed by Wilson, Hawthorne, and Tsou (1951). It states that there are at least two components of mitotic movement; a dipolar cytoplasmic orientation, and a nuclear component inherent in the chromosome or its kinetochore. A simpler theory, such as this is, is much more readily and easily used as a basic working hypothesis.

The Effects of Terramycin-HCl

The primary cytological effects of terramycin-HCl are on prophase and "antephase." Therefore, this antibiotic may be classified as a "prophase-poison" or better still, a "pre-prophase-poison." That movement of chromosomes from prophase to subsequent stages is stalled or prevented, is shown by the early increase in the proportion of late-prophases to total number of dividing cells. As time and concentration is increased, this effect is even more noticeable, as indicated by the graphs in text Figures 2, 3, 5, 7, 10, and 12.

Chromosomes which are in early-prophase and "late-antephase" at the time of treatment, probably never proceed farther than late-prophase. This may well be due to an inhibitive action of terramycin-HCl on the center and kinetochore

movements. The movement of the chromosomes, which is realized, may be caused by forces of contraction and torsion within the chromosomes themselves. That they are incapable of forming a pro-metaphase clump, is clearly shown by the early disappearance of this stage. The morphological changes are not interfered with, except for a possible slowing action. Therefore, the continued morphological changes along with the inhibition of movement, can be considered to account for severely contracted and reverting chromosomes in late-prophase. The degree of contraction at the time of reversion, may be related to the advancement of prophase at the time of treatment. This conclusion is drawn on the basis that the number of contracted figures and degree of contraction increases with time and also shows a correlation with concentration and length of treatment period.

"Antephase," according to Bullough (1952, p. 145), "is undoubtedly the most sensitive phase in the entire course of a division." The action of terramycin-HCl on "antephase" is a preventative one. This is clearly illustrated by the reduction and subsequent disappearance of early-prophases in division, during the early hours of treatment in continuous treatment, and in recovery following short time treatments. The failure of new cells to enter into division accounts for the reduction in the mitotic indices as is shown in text Figures 1, 4 and 9. That recovery from such inhibition

may occur is shown by the later return of early-prophase during recovery. It is interesting to note, that in the short time treatment of 15 minutes with concentrations of 100 and 200 ppm., the increase in number of early-prophases and the mitotic index is realized within three to four hours, which is considered to be the normal period of time a cell requires to go through mitosis.

The cytological effects of this antibiotic on post-prophase are not as pronounced as on prophase. The slightly scattered metaphases may possibly be considered as normal and due to a slowing action on the movement of chromosomes from the metaphase plate, thus making it possible to see them. Or, they may be considered to be the result of a partial impairment of the spindle. Likewise, spread anaphases and telophases may be considered as a normal condition, made observable by slow movement. On the other hand, a partial impairment of the spindle and kinetochore repulsion could account for the spread, ball-shaped groupings.

Severely scattered metaphases probably come from chromosomes which were in late-prophase at the time of treatment. This is indicated by their arrangement within the cell and their later appearance in treatment and recovery. This type of cell rarely enters into anaphase, and usually reverts *in situ*. Theoretically, this should lead to four-stranded chromosomes. It may be assumed that these cells, as well as

other cells undergoing mitosis at time of treatment, are incapable of redividing. This conclusion is made on the basis that polytene or four-stranded chromosomes have never been observed in recovery material. The lack of side-roots in the immersion region of roots subjected to continuous treatment, may be considered as further evidence. However, the exact disposition of these cells is not known and can only be speculated on at the present time.

The bridges and fragments, which were observed in anaphase and telophase, were of the type reported and pictured by Tanaka and Satô (1952), and Levan and Tjio (1951). A close examination of an equal number of control slides, showed that there was no significant increase in occurrence or kind. Therefore, it does not seem possible to conclude that such bridges and fragments are the result of treatment. It is more likely that they are caused by an entanglement of chromosomes with trabants. Also, there was no evidence of ring fragments or translocations which are commonly associated with radiomimetic chemicals such as the mustard gases (Auerbach, 1949). Such fragmentation as has been noted does not seem to be of a degree or kind which would warrant classification of this antibiotic as a mutagenic agent.

Terramycin-HCl as Compared to Colchicine

The cytological effects of terramycin-HCl are quite different from those of colchicine. Colchicine has a stalling effect on pro-metaphase and metaphase, which ultimately leads to unipolar anaphases and telophases causing polyploidy. This early, complete disruption of spindle function is not caused by terramycin-HCl. Colchicine stalls anaphase and prevents cytokinesis, forming binucleate cells. Terramycin-HCl exhibits only a very slight effect of this type, as is indicated by the appearance of only a few binucleate cells. The severely scattered metaphases from colchicine treatment go through anaphase and telophase, and multinucleate cells are formed. Following terramycin-HCl treatment, severely scattered metaphases revert in situ and do not lead to multinucleate cells. In contrast to terramycin-HCl, colchicine has a stimulatory effect on "antephase," rather than an inhibiting one. Further division of colchicine-effected mitotic cells has been observed in recovery. There is no evidence that mitotic cells effected by terramycin-HCl redivide in recovery material.

Cytological and Antibiotic Activity of Antibiotics

The cytological effects of terramycin-HCl have followed the same pattern as shown by all the other antibiotics which

have been studied by the cytology group at Michigan State College. These effects in review are: a high percentage of reverting prophases; a prevention of the movement of prophase chromosomes into post-prophase and a pile-up of late-prophases; an inhibiting effect on "metaphase" resulting in a lowering of the mitotic index; the occurrence of several scattered metaphases; and failure to find four-stranded chromosomes in recovery material. This would seem to indicate that there may be a direct correlation between antibiotic and cytological effects. It is possible that the action of an antibiotic on sensitive bacteria is due to an inhibiting effect on the division of the bacteria. Such failure of reproduction would lead to their death.

The only difference, which has been found through the cytological study of the action of antibiotics, is in the concentration level at which effects and toxicity are realized. In connection with this relationship, it should be noted that Actidione shows the most toxicity as an antibiotic and antimitotic. It is the only antibiotic, thus far studied, which is too toxic for use in medical treatments. The concentrations of Actidione, which show cytological effects and toxicity, are much lower than for the medically accepted antibiotics. In this connection, Dr. G. B. Wilson has noticed a direct correlation between antibiotic and cytological toxicity ranges, insofar as they had been studied. If these observations

should prove to be valid, it would seem to indicate that the toxic effects of new antibiotics could be subjected to a preliminary cytological screening test.

SUMMARY

1. The Pisum test was used to determine the cytological effects of terramycin-HCl. Root tips of Pisum sativum were subjected to eight hours continuous treatment with concentrations ranging from 10 ppm to 200 ppm; and for short time treatments for 15 minutes with 100 ppm and 200 ppm and for 30 minutes with 600 ppm and 800 ppm, followed by a recovery period in nutrient solution.
2. The mitotic index decreased according to time and concentration. Early prophases disappeared and there was a pile-up at late prophase. Scattered and spread figures were observed in post prophase. Continuous eight hour treatment inhibited side-root development.
3. It was concluded that: terramycin-HCl prevents "antephase" from entering into mitosis; may have a slight destructive effect on the spindle; interferes with center and kinetochore movement in prophase; prevents cells in mitosis at the time of treatment from redividing. No mutagenic action is indicated.
4. The cytological effects of terramycin-HCl and colchicine are different in several respects: a) the former has an inhibiting effect on "antephase," whereas, the latter

is stimulating; b) the former does not seriously effect post-prophase, whereas, the latter does; c) the former inhibits effected cells from dividing again, whereas, the latter does not.

5. The cytological effects of all antibiotics, thus far studied, follow the same pattern. There is some indication of a correlation between cytological effects and the action of antibiotics.

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PLATE I

Figure 1 Root development of seedlings, after treatment with terramycin-HCl and six days of "paper towel" recovery.

A - Treated with 200 ppm for eight hours.

B - Treated with 100 ppm for eight hours.

C - Control.

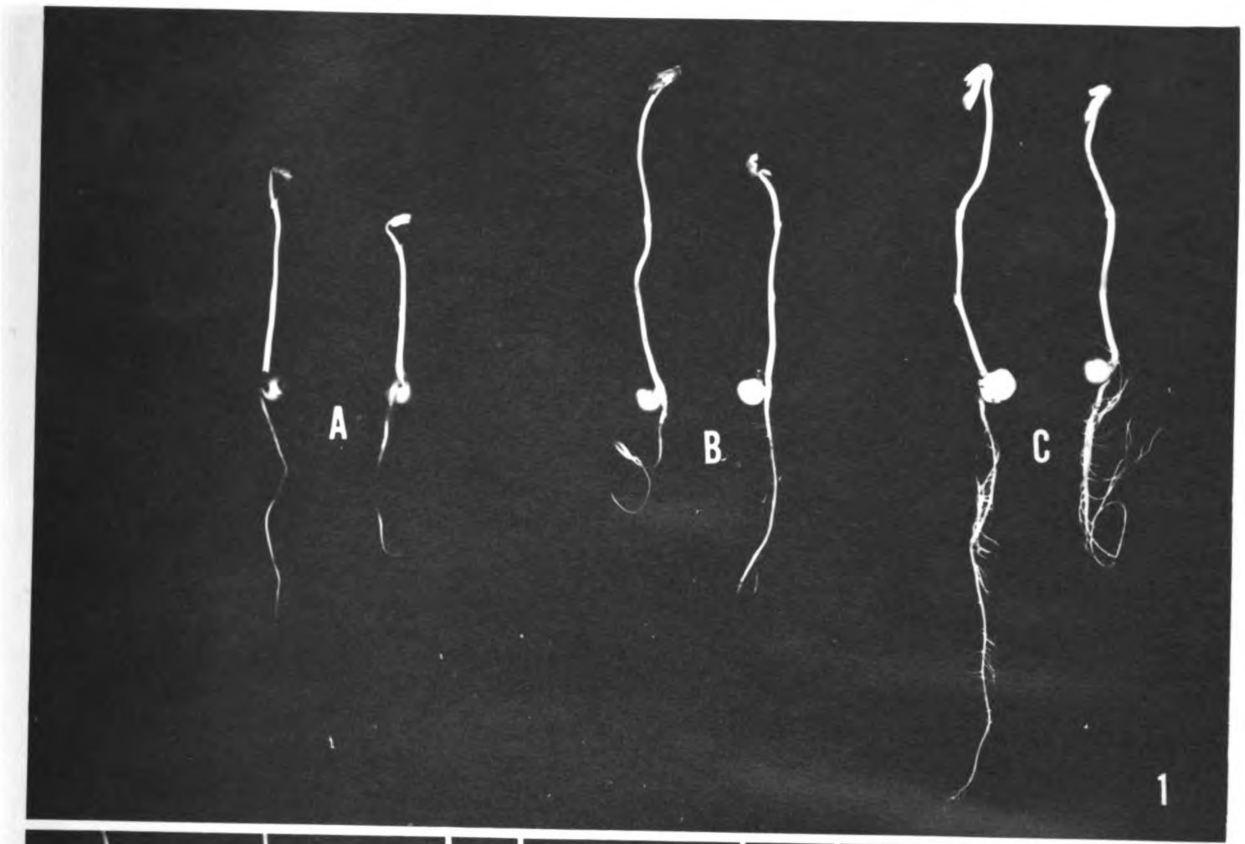
Figure 2 Root development of seedlings, after treatment with terramycin-HCl and ten days of "paper towel" recovery.

A - Treated with 50 ppm for eight hours.

B - Treated with 25 ppm for eight hours.

C - Treated with 10 ppm for eight hours.

D - Control.



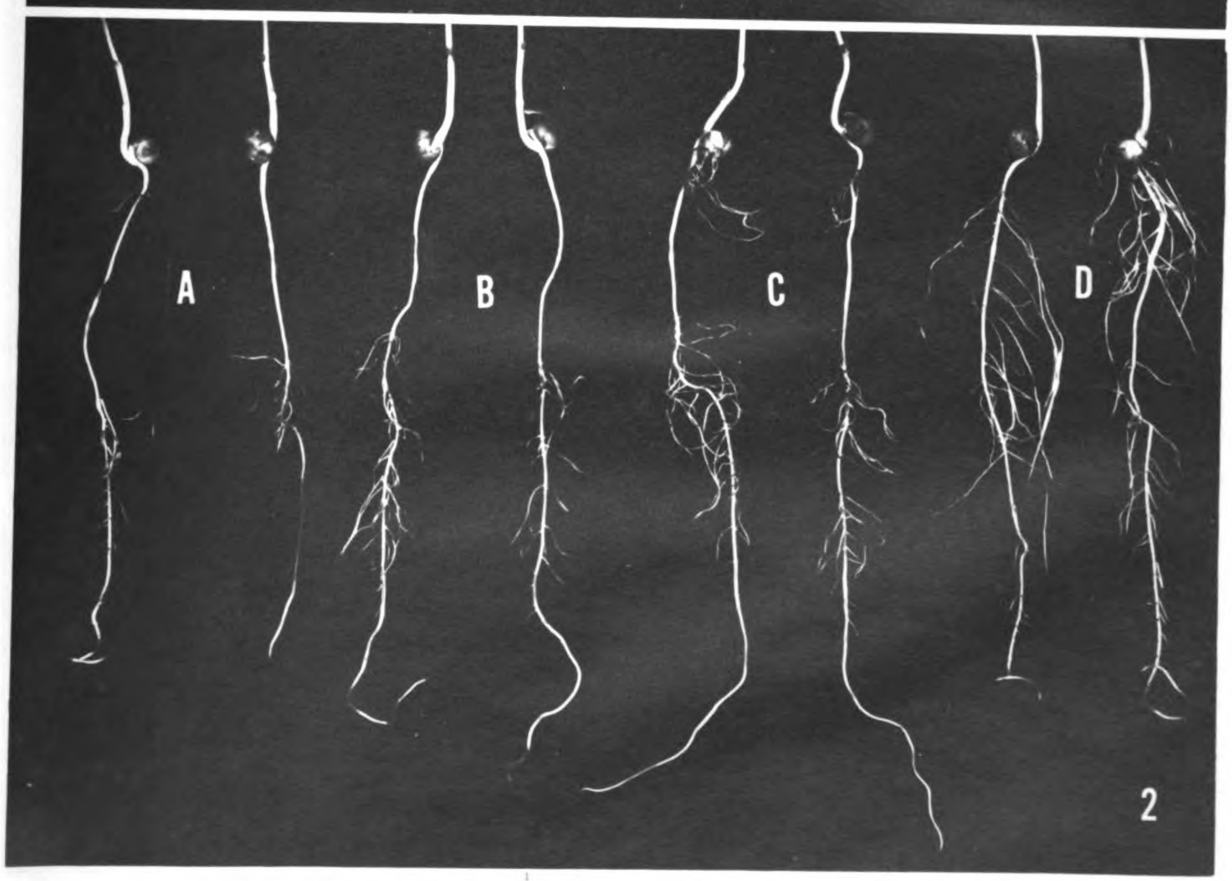


PLATE I

PLATE 11

Fig. 1 Interphases

Fig. 2 Early prophases

Fig. 3 Late prophase

Fig. 5 Contracted late prophase, prometaphase and early prophase

Fig. 6-7 Contracted late prophase

Fig. 8 Prometaphase

Fig. 9 Prometaphase with lagging chromosome

Fig. 10 Prometaphase and early anaphase

Fig. 11 Early metaphase and prometaphase

Fig. 12 Early and late prophase; early metaphase

Fig. 13-14 Metaphases

Fig. 15 Early prophase and anaphase

Fig. 16 Telophase with lagging chromosome

Each division of the scale represents ten microns.

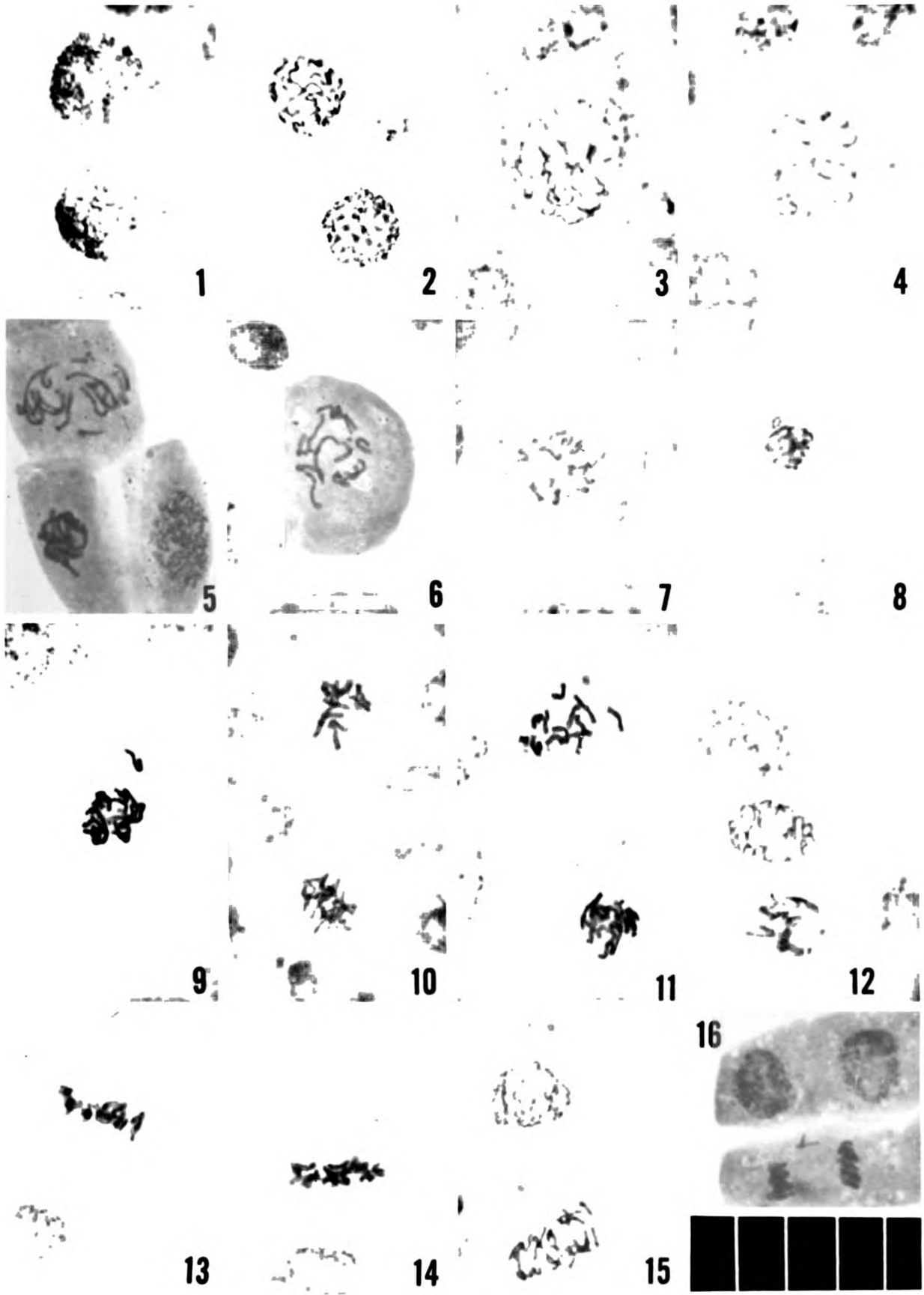


PLATE II

PLATE III

EFFECTS OF TERRAMICIN-HCl ON *PISUM SATIVUM*

Concentrations and type of treatment are indicated at the bottom of this page.

Fig. 1 Reverting mid-prophase; (1), 1/4 hour recovery

Fig. 2 Reverting late and mid prophases; (2), 1/2 hour recovery

Fig. 3 Reverting slightly contracted late prophase; (2), 4 hour recovery

Fig. 4 Reverting moderately contracted late prophase; (2), 7 hour recovery

Fig. 5 Moderately contracted late prophase; (3), 4 hour recovery

Fig. 6 Severely contracted late prophase; (2), 7 hour recovery

Fig. 7 Reverting severely contracted late prophase; (2), 5 hour recovery

Fig. 8 Slightly scattered metaphase; (1), 1/4 hour recovery

Fig. 9 Severely scattered metaphase; (1), 1/2 hour recovery

Fig. 10 Reverting severely scattered metaphase; (3), 4 hour recovery

Fig. 11 Anaphase with bridge due to entanglement of trabants; (2), 3 hour recovery

Fig. 12 Anaphase with lagging chromosomes due to entanglement of trabants; (1), 1/4 hour recovery

Fig. 13 Spread anaphase; (2), 4 hour recovery

Fig. 14 Spread anaphase; (1), 1 hour recovery

Fig. 15 Spread telophase; (3), 3 hour recovery

Fig. 16 Telophase with bridges; (3) , 1 hour recovery

(1) Mitoses from 200 ppm treatment for 15 minutes
(2) Mitoses from 600 ppm treatment for 30 minutes
(3) Mitoses from 800 ppm treatment for 30 minutes

Each division of the scale represents ten microns.

PLATE III