CYTOLOGICAL MEASUREMENTS OF C-MITOTIC AND PROPHASE POISON ACTIONS

By

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AN ABSTRACT

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The Pisum test was used in an endeavor to set up cytological criteria for c-mitotic and prophase poison actions. Colchicine was chosen as the standard c-mitotic agent, and actidione, an antifungal antibiotic as the standard prophase poison agent.

Dividing cells in the root tip meristematic region of Pisum when treated with colchicine in varying doses, showed the usual scattered and clumped configurations usually associated with this drug. It is suggested that the scatters are due to a "dilution effect." The clumps apparently are due to a full destruction of the spindle or its precursor. If certain weights are assigned the colchicine-produced configurations, a series of linear relationships are obtained on a log of time versus a log of dose basis for various index intercepts. The cytological characteristics which a drug or chemical must exhibit to be classed as a c-mitotic agent are listed.

If meristematic tissue of Pisum is subjected to actidione treatment, two types of effects are exhibited. First, an inhibition of new mitoses is manifested by the drop in mitotic index, and second, an anomalous prophase configuration is evident. On the basis of the above, three types of measurements were performed: (1) Measurement based on a ratio of certain configurations, (2) Measurement based on a configuration (actidione prophase), (3) Measurement based on a combination of (1) and (2) above.

A straight line relationship is achieved for each type of measurement when the times at which the various dose curves cross arbitrarily chosen ratio or percent intercepts are plotted on a log of time versus a log of dose basis. Since all the linear relationships have slopes which are not statistically different, a line with a slope which is the average of all the previously obtained slopes can be determined which indicates that all three measurements must measure the same thing. The cytological characteristics which a drug or chemical must exhibit to be classed as a prophase poison are listed.

While colchicine appears to act upon some mechanism occuring from late prophase to metaphase, actidione appears to act on a mechanism occuring from interphase through mid-prophase.

Important implications are evident in the action of a drug such as actidione which can control the onset of mitosis, the most important of which would be in the field of anti-carcinogens.

The importance of timing in the movement cycle of mitosis is discussed in connection with the apparent actions of colchicine and actidione. A diagrammatic representation of the colchicine and actidione pathways through the mitotic cycle has been presented.

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INTRODUCTION

The precision of the mechanism of mitosis makes it a remarkable phenomenon. It is basic to all branches of biology, whatever the discipline. Yet in the 75 years since Flemming first recognized the significance of this mechanism, it is sobering indeed to reflect upon the fact that we know almost as little as did Flemming at the time of his discovery. W. D. W. Thompson (Hughes 1952) says, " We have learned many things about cell division, but we do not know much in the end."

Mitosis is of a cyclic nature, the arbitrary phases of which have been erected by biologists for their own convenience. For many years cytologists have used various means to interrupt this cycle as tools in studying the mechanism and trying to understand the underlying problems of mitosis. Among these tools are certain antimitotic agents, both physical and chemical. They range all the way from ultrasonics to ordinary table salt. The number of investigators who have indulged in this sort of thing in recent years, and the literature which they have produced has become prodigious indeed. The theories and ideas on the mechanism

of mitosis and how it is affected by various chemical and physical agents have come to be almost directly proportional to the number of investigators engaged in such studies. Yet, in spite of the tremendous amount of work which has been done on the mechanism of mitosis (or because of it) and the resulting apparent conflicts, certain basic truths are evident with respect to chemical interruption of the mitotic cycle:

(1) Some chemicals are more efficient than others.

(2) The effects of some chemicals are easily recognizable as being associated with specific cytological aberrations, and are repeatable under standard conditions.

As is true with most studies in which the investigator must observe a result and come to a decision, there is a great deal of subjectivity involved in experiments using antimitotic agents. This in turn has led to a lack of standardization of terminology. Cytological effects which one investigator might call pertinent, another will ignore completely, and vice versa. This confusion which permeates the whole field of antimitotics has led to a confusion as to basic action. For many years now, any chemical which produces an effect on the mitotic mechanism which even vaguely resembles that produced by such well known drugs as colchicine has been labeled immediately as "c-mitotic." This holds even in cases where it is obvious that the action

of the drug is on a mechanism entirely different from that upon which colchicine acts. In spite of many recent investigations which have pointed out that there is not necessarily a common mode of action to produce common cytological configurations (Allen, Wilson and Powell 1950, D'Amato 1948b, Guttman 1952, Hindmarsh 1952), many investigators still cling to the classical interpretation of c-mitotic action. Levan (1954) continues to defend his concept of c-mitotic action and states that ". . . all morphologically discernible disturbances of spindle function irrespective of any notions as to the mode of action underlying the effects. . ." must be described as c-mitotic.

The cytology group at Michigan State University has attempted for several years to categorize certain chemicals on the basis of their action on the mitotic mechanism. The present investigation is but a small part of this whole project, and is based on an original premise established by D'Amato (1948b) that certain chemicals are, in fact, prophase poisons rather than c-mitotic agents, even though some of the cytological pictures produced resemble c-mitosis. An endeavor has been made to establish that a typical c-mitotic agent (colchicine) acts on a different mechanism and at a diffenent time in the mitotic cycle than does a typical prophase poison (actidione). The drugs were chosen for the reasons previously stated: (1) Both are efficient, and (2) both produce cytological effects which are easily

recognized and reproduced.

The objectives of this study may then be enumerated as follows:

(1) To demonstrate that colchicine, a typical c-mitotic agent, acts through a different mode of action and at a different time than does actidione, a typical prophase poison.

(2) To establish a standard procedure for matching future drugs and chemicals to the category in which they appear to fall cytologically.

(3) To establish that the cytological effects observed are indeed a valid measure of the effect of the drug or chemical.

(4) To establish, if possible, a time-dose relationship with regard to cytological effect.

LITERATURE REVIEW

The name colchicine extends back into the mists of antiquity. Not only do modern formularies list Colchicum, the producer of the pure substance colchicine, but this plant is probably one of those mentioned in the Ebers Papyrus, an Egyptian document prepared about 1550 B.C. In the first century Dioscorides mentioned its toxicity, and his diagrams of the plant Colchicum were so good that they were copied by scholars for 1500 years. Colchicine is an alkaloid found in certain members of the Liliaceae, especially in species of Colchicum. Colchicum autumnale , meadow saffron or autumn crocus, is native to the old World. Specifically it is indigenous to southern, central and western Europe, east to the Balkans, southern Russia and Kashmir. It is likewise found in North Africa.

It was not until 1887 that the alkaloid in its more or less pure form was isolated by Houde (Dixon and Malden, 1908). Pernice in 1889 (Eigsti, Dustin, Jr. and Gay-Winn, 1949) was the first to observe the cytological effects of colchicine. He is reported to have noticed the large number of cells in division, and also to have noticed that

there were a large number of abnormalities present. It appears that Pernice was one of the first to note the blocking action of colchicine, since he claimed that the stages after metaphase were rarely seen. A decade and a half passed before similar observations were made by Dixon (1905). He reported disturbing effects of the drug on the mitosis of leucocytes. Later Dixon and Malden (1908) reported that colchicine initiated the appearance of erythroblasts in mammalian blood. After injection of colchicine into rabbits, rats, dogs, and humans, there was a transient leucopaenia followed about an hour later by marked leucocytosis, mostly polymorphonucleocytes. Dustin (1934) and Lits (1934), both using mice as the test organism, advanced the idea that colchicine was actually stimulating mitosis, thus accounting for the increased number of dividing cells. However, Ludford (1936) came to the conclusion that the increased number of mitoses was actually due to an accumulation of figures resulting from a blockage in the formation of the mitotic spindle. This was later confirmed by Brues and Jackson (1937) in their work with mouse hepatic tissue.

A tremendous impetus was given colchicine research when it was reported by Blakeslee (1937), Blakeslee and Avery (1937), and Dustin, Havas, and Lits (1937) that the drug was capable of producing polyploidy in plants. Indeed, the flood of reports which followed in the next decade is well illustrated by the bibliographies prepared by Eigsti

(1947) and Eigsti and Dustin, Jr. (1949). Cytologists now became interested in how the drug caused polyploidy. Nebel (1937) and Nebel and Ruttle (1938), in studies made in the living stamen hairs of Tradescantia reflexa, reported that while colchicine treatment inhibited the formation of the achromatic figure, all other nuclear developments were allowed to continue. Thus it followed that with the absence of an anaphase separation, a telophase nucleus was formed which was tetraploid. Derman (1938) confirmed the conclusions of Nebel and Ruttle in his work on Rhoeo discolor stamen hair cells. Many other investigators in the field (e.g., Beams and King 1938, Eigsti 1938, Walker 1938) soon reported results using plant tissues which confirmed the previously reported observations and conclusions. Beams and King, and Eigsti, besides reporting the observation of polyploidy, also found micronucleated and binucleated cells. Beams and King likewise were able to produce results similar to that of colchicine treatment by using low temperature (3° C) treatments. They concluded that colchicine lowered the viscosity of the cytoplasm and prevented the normal viscosity changes that accompany normal mitosis. Thus the polar centers and the spindle, which they felt were cytoplasmic in origin, were not formed and the nuclear cycle continued while the cytoplasmic cycle was blocked.

Levan (1938) introduced what is now known as the "Allium test." This test has become a standard cytological

procedure throughout the world for determining the effects of all sorts of drugs and chemicals on actively dividing meristematic tissue. According to this method onions are rooted by placing them over beakers of water such that just the end of the onion projects into the solution. These growing roots were then transferred to various concentrations of colchicine for varying periods of time. At regular intervals cuttings were made and prepared for cytological examination. The bulbs with roots left on them were returned to water after the treatment time had ended. Levan was of the opinion that the effect of colchicine was a specific one, and he called this "c-mitosis." He defined c-mitosis as an inactivation of the spindle in conjunction with a delay in the separation of the kinetochores. This resulted in a completion of the chromosome mitosis without a completion of nuclear or cytoplasmic mitosis. Levan was unable to observe any effects of colchicine on the prophase of mitosis. He described the chromosomes as reaching metaphase morphologically but not spatially. The configurations came eventually to resemble a diakinesis-like picture with the chromosomes spread all over the cell. Since the falling apart of the chromatids at the kinetochore was so long delayed, there appeared to be a build-up of highly contracted, diakinesis-like metaphases. These configurations Levan called "c-pairs." After the passage of a certain amount of time, the kinetochores eventually fell apart, not however,

always at the same time. Even though the kinetochores divided, they did not seem to move apart, and a new interphase nucleus was formed which included all these chromatids, thus a tetraploid cell resulted. Levan was able to demonstrate that this destruction of the spindle apparatus was reversible. Upon re-immersing the roots in water after treatment, the spindle gradually started to form. The most immediate results observed upon recovery were the formation of odd-shaped nuclei, multipolar cells, and microcytes. Gradually more and more normal bipolar divisions appeared, until finally all were normal. In later investigations Levan (1939, 1940) further supported his conclusions. In this later work he observed that the exterior spindle (centrosomic) and the interior spindle (centromeric) react differentially to the drug. When the concentration of colchicine was just above the threshold value, the exterior spindle was inactivated first. This was manifested cytologically by a cessation of the chromosome movement cycle. The delay in the separation of the kinetochores was associated with an inactivation of the interior spindle. This was manifested cytologically by the accumulation of c-metaphases in the tissue.

Shimamura (1939), using the Allium test, obtained results close to those of Levan. He noticed that the "c-pairs after kinetochore separation were arranged around a spherical, refractile-type body. He assumed that this was

the remains of the degenerating spindle. A similar refractile body was noticed by Gaulden and Carlson (1951). Shimamura also observed binucleate cells in which the two nuclei were in the process of merging to form a dumb-bell shaped structure.

Bhaduri (1939) studied the effects of different forms of colchicine on the growing roots of Vicia fabia, the broad bean. His sources of colchicine were the corm and seed, the pure alkaloid, and colchicine salicylate. He suggested that there might possibly be an effect on the nuclear membrane. The spindle collapsed under all three forms of the drug, with the resultant formation of tetraploid nuclei and multinucleated cells. Bhaduri believed that colchicine was possibly a catalyst which caused chemical reactions that do not ordinarily take place.

Shimamura (1940) used centrifugal forces to study certain aspects of nuclear division. In this investigation he terms the spindle the "Atractosome." He offers evidence that this is not formed in those cells treated with colchicine. He further asserts that the chromosomes during mitosis do not lie in the cytoplasm directly, but rather in the "Atractoplasm." Soon thereafter, Wada (1940) concluded that the effect of colchicine was specific for the "Atraktoplasm," and this specificity manifested itself as a decrease in its surface tension. This in turn stopped the movement of the chromosomes into the metaphase and

anaphase configurations. Wada postulated that colchicine affected the atractoplasm in such a way that it was turned into cytoplasm.

Hawkes (1942) studied the effects of colchicine on onion seedlings. He used a concentration of 0.04%. His results were similar to those of Levan with one major exception. Levan felt that the c-pairs fell apart several hours after initial onset of c-metaphase, whereas Hawkes observed that they do not fall apart until the subsequent interphase. He also postulated that the division of the chromosomes is approximately twice as long under the influence of colchicine. His control cycle took about 16½ hours while the colchicine treated cycle took about 33 hours. He did not report the presence of clumps in his material.

Cornman (1942) studied the effects of colchicine on the excised roots of Colchicum byzantinum. It is well known that the alkaloid colchicine will not affect mitosis in actively dividing tissue of the genus Colchicum. But if the roots are excised, effects are noted. Cornman arrived at conclusions similar to those of previous investigators. He felt that binucleate cells with bridged nuclei were the result of a cessation of anaphase movement and subsequent return to interkinesis without cytokinesis.

Berger and Witkus (1943) compared the effects of colchicine on Allium with its effect on Spinacia. In regard to the Allium, their results were in line with those

of earlier investigators. They also noted the so-called "achromatic sphere" or hyaline substance reported by Shimamura. Spinacia, however, produced results quite different from those of Allium. No achromatic sphere was observed, and diplochromosomes formed dense clumps at c-metaphase. They felt that the very irregular restitution nuclei formed with Allium after treatment were due to the presence of the achromatic sphere. Since no sphere is formed in Spinacia and the restitution nuclei following treatment were always very spherical. They stated that "the chromosomes of Spinacia during the reversion phase do not resemble anaphase or telophase chromosomes, but go through a stage similar to the prochromosome stage of insect spermatogenesis."

As a result of all this activity in the first five or six years following the report of Blakeslee that the drug produced polyploidy in plant cells, colchicine has come to be regarded as the standard substance with regard to the initiation of c-mitosis. Thus the terminology used in colchicine research has been carried over to describe the effects of other drugs, the superficial appearance of which tend to parallel those of colchicine.

Levan and Östegren (1943) initiated a comparative study of the effects of colchicine and some of the naphthalene series. In this study they arrived at the conclusion that the action of these drugs was due to their physical

properties rather than to any special chemical attributes. This assumption was based on their observation that there was a negative correlation between c-mitotic activity and water solubility of the drugs used. This fact, in turn, suggested to them that the mode of action might possibly reside in the lipoid phase of the cell. Similar experiments the same year (Östegren and Levan, 1943) using benzenes produced similar results. Following this, Östegren (1944) compared the colchicine type of mitosis with the narcotic effect of several other related compounds. In plants these narcotic effects were c-mitosis, c-tumor formation, chromosome contraction, and toxicity. In this connection Östegren postulated that the mechanism whereby colchicine acted was through association with the lipophilic side chains of the polypeptide protein chains. This association was presumed to disturb the degree of folding of the polypeptide chains, thus the fibrous protein molecules became changed into a more or less corpuscular shape.

Steinegger and Levan (1947) attempted to demonstrate that the c-mitotic activity of a substance resided in its thermodynamic activity. In a follow-up investigation (1948) they purported to show that the "C" ring of colchicine is of decisive significance for specific c-mitotic activity in Allium.

The higher plants have not been the only test organisms used for colchicine investigations. Certain animals,

mostly cold-blooded have also been treated with colchicine. Barber and Callan (1943) used the newt Triturus viridescens for experimentation. Their observations paralleled those of previous investigators who used plant tissue. A blockage of metaphase was noted, but no effect on prophases was observed. They observed star metaphases, distorted star metaphases, exploded metaphases, and ball metaphases. The mitotic abnormalities noted suggested the tactoid theory of spindle formation as postulated by Bernal. They concluded that the exploded metaphases were a result of inactivation of the centromeres, the star metaphases were the result of inactivation of the centrosomes, and the ball metaphase was the result of inactivation of both.

Peters (1946) made studies on the effects of colchicine on the cornea of the newt. He distinguished two types of abnormalities. The first of these was such that there was no orientation of the spindle attachment regions and the chromosomes were either clumped or scattered. The second abnormality observed was of the type with the spindle attachment regions orientated to one another and the chromosomes forming one or more stars or a normal metaphase. Since he was unable to observe chromosomal fibers in the unoriented metaphases, but was able to observe some (although discontinuous) in the star metaphases, he concluded that the star formations were due to the action of chromosomal fibers and centriole, both of which acted independently of the continu-

ous fibers. He also assumed that the chromosomal fibers recovered first from the effects of colchicine.

Following World War II interest in colchicine was renewed. D'Amato (1948a) used Allium which had been stored at 0-4°C. This low temperature had produced sticky chromosomes. He observed partial c-mitosis (scatters) and full c-mitosis (ball metaphase). He felt that the ball metaphases were a subvital effect signifying poisoning. He further postulated that the scatters and clumps were due to (1) a time factor, and (2) a physiological factor. D'Amato also observed the "exploded metaphases" of Barber and Callan, the arrangement of c-pairs around a spherical surface, and precocious reversion processes which he attributed to the peculiar physiological conditions in which the colchicine action had developed.

Sigenaga (1949) concluded that the action of colchicine was due to its effect on the protoplasm which led to a breakdown of the visco-regulating mechanism of the chromosomes and spindle, whereas Wada (1949) postulated that the disturbance of the submicroscopic structure of the atractoplasm was the principle effect of colchicine. Observations on the living spindle in Chaetoptera by use of the polarizing microscope were made by Inoué (1952). These observations showed that colchicine caused the spindle to contract, from which he concluded that there had been a disorientation of the micelles of the spindle.

The tremendous number of theories which have been advanced concerning the mode of action of colchicine only tend to reenforce the idea that some distinction needs to be made between the true c-mitotic agents in the restricted sense of the word (i.e., spindle interruption leading to polyploidy) and the agents which produce results only superficially similar to those of colchicine. D'Amato (1948b) called attention to the fact that many substances which were thought to cause c-mitosis were, in effect, really prophase poisons. Later (1949) he pointed out that,". . .the spindle inhibiting effect manifested by typical c-mitotic poisons may be of quite another nature than that by which preprophase poisons induce c-mitosis. . ." D'Amato and Avanzi (1949a) substantiated D'Amato's previous investigations (1948b). They concluded that ". . .typical c-mitotic agents are only those which induce c-mitosis but do not inhibit mitosis." On the other hand a typical prophase poison inhibits the onset of new mitoses (D'Amato and Avanzi 1949b).

Others also developed an interest in this distinction between the two kinds of chemical agents. For example a comparative study was made between the effects of the salts of nucleic acids and colchicine by Allen, Wilson,and Powell (1950) in which the authors concluded that the "reductional groups" (Huskins 1948) observed after sodium nucleate treatment represented a different nuclear response than did the "distributive c-mitoses" formed during colchicine (D'Amato

1948a) or vitamin K treatments (Nybom and Knutson 1947). However Levan and Lotfy (1949) using naphthalene acetic acid in the Allium test had concluded that "reductional groupings" were nothing more than modified c-mitotic configurations. Hindmarsh (1951) studied the effects of some nitro-phenols on mitosis. From this investigation she concluded that the term "c-mitosis" was much too broadly applied. She said, "Many authors either assume without justification that substances which suppress spindle formation have a common mode of action, or do not recognize that, by use of this term, they imply a common mode of action for all such substances. . ."

Working with Allium cepa and Vicia fabia, Levan (1951) set up three categories into which cytological reactions could be put on the basis of morphological types. They were (1) the lethal and toxic reactions, (2) the reversible physiological reactions, and (3) the mutagenic reactions. The action of colchicine is represented by category (2). A similar categorization had been made by Loveless and Revell (1949). On the basis of their investigation, they placed all "mitotic poisons " into three classes: (1) radiomimetic, (2) toxic action during interphase, and (3) c-mitotic agents.

Many antibiotics have been studied (Wilson 1950a, Wilson and Bowen 1951, Hawthorne 1951, Hawthorne and Wilson 1952, Huston 1952, Bowen 1953, Bowen and Wilson 1954, and the unpublished report of Miss S. Wilson) with results indi-

cating that although their effects are often superficially similar to colchicine, there is no evidence that any of them are "c-mitotic agents." Certain agriculturally important chemicals, including Endothal (Daniel 1953) and technical Lindane (Tsou 1954) give widely different results when subjected to the "Pisum" test. Loosely, these might be termed "c-mitotic." Since then Endothal has been proven to be mutagenic (Wilson,Daniel and Wilson 1956; Hadder, unpublished). Other work by Wilson and associates (Wilson, Hawthorne, and Tsou 1951, Wilson, Tsou, and Hyppio 1952) on many variations in mitosis has further pointed up the need of a quantitative investigation on some type substances in an effort to categorize the many drugs and chemicals which affect mitosis.

Hawthorne (1951) and Hawthorne and Wilson (1952) investigated the effects of the antifungal, antibiotic actidione on mitosis. Concentration of 1 ppm to 80 ppm were used, both as short time treatments and as continuous treatments. It was observed that actidione acted in a manner which placed it in the class of the prophase poisons as defined by D'Amato (1949). Prophase did not progress beyond the late prophase stage even at the lower doses. Upon being arrested at late prophase spatially, the chromosomes appeared to continue their morphological cycle in situ, eventually becoming telomorphic. The higher the dose the sooner the prophase-poisoning effect appeared. It was also observed

that the drug did not allow new mitoses to be initiated. Thus there was also some effect on the preprophase stages. After four to six hours very few division figures were observed. The authors concluded that although some of the configuration found superficially resembled those of colchicine, this drug is indeed a prophase poison in the strict sense of the term.

Bowen (1953) and Bowen and Wilson (1954) studied the cytological effects of some antibiotics and compared these effects with those of colchicine. They observed an increase in the absolute numbers of prophases over postprophases during treatment, from which they suggested there might be a stimulation of mitosis by colchicine. Bowen stated ". . . that the impairment of the spindle . . .is not an all or none process." This is shown by the fact that disorganized postmetaphases and micronuclei are achieved with threshold doses of the drug.

On the assumption that a re-analysis of the mechanism whereby colchicine affected mitosis was needed, Hyppio (1954) and Wilson and Hyppio (1955) undertook an exhaustive investigation. Among other things, they concluded that colchicine, besides affecting the functioning spindle, also affects the pre-prophase kinetochore such that prophase movement is impeded. This results in the so-called "scatters" which later give rise to multinucleated cells and microcytes. This is termed as ". . .action on the

antephase." The investigators also concluded that ". . . the complex cytological effects of colchicine make it a much less efficient polyploidizing agent than popularly supposed."

In 1954 Levan reported the results of some work on the effects of colchicine on mouse ascites tumors. The observations were more or less in accord with those he had found in Allium (Levan 1938). However, in this case he found that the chromatids of the c-pairs did not separate until interkinesis following the c-mitosis. He also distinguished between "initial c-mitosis" as represented by balled and clumped metaphases, and "scattered c-mitosis" as represented by the highly scattered c-pairs. Levan defended his concept of c-mitosis, and stated categorically that ". . .the term should be applied to all morphologically discernible disturbances of spindle function irrespective of any notions as to the mode of action underlying the effects. . ."

A recent addition to the colchicine literature is a lengthy volume by Eigsti and Dustin, Jr. (1955) in which is gathered together a comprehensive review of much of the colchicine work which has taken place in the last two decades, as related to agriculture, medicine, biology, and chemistry.

MATERIALS AND METHODS

Experimental Procedure

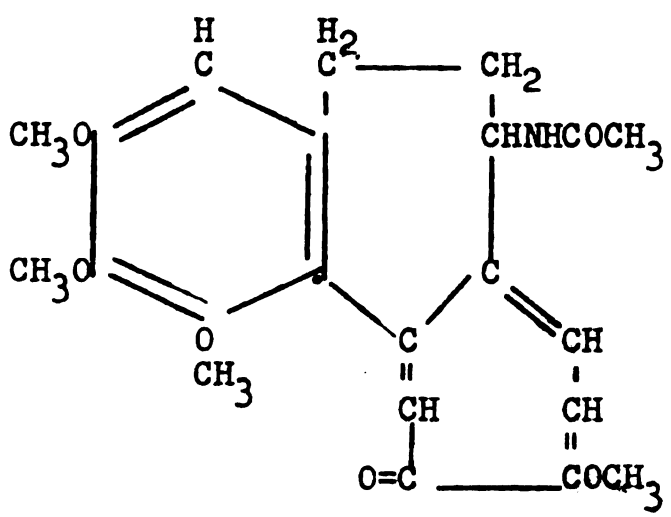
The meristematic tissue used in these studies was the actively growing root tip of *Pisum sativum var. Alaska*. The peas were kindly furnished by the Ferry-Morse Seed Company who stated that they were disease-free and of relative genetic homogeneity. The company also stated that this variety had not been treated with any antifungal agents.

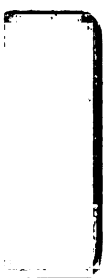
The peas were soaked in distilled water in chemically clean beakers for four to six hours or until they were fully imbibed with water. They were then rolled in paper-toweling wet with distilled water, and the towels were placed upright in beakers in the bottom of which was about an inch of distilled water. The peas were allowed to germinate at room temperature (about 25°C). It was generally found that the pea seedlings had attained a length of two and a half to three and a half centimeters on the third day after rolling. Upon attaining this length the seedlings were collected and treated.

Seedlings which had attained the proper length were

examined macroscopically for visible anomalies, and if free from these were inserted into a grid of paraffined rat wire which was molded over the top of a 250 ml. beaker in which was contained the test solution. These solutions were aerated by blowing air from a laboratory outlet through a cotton and charcoal filter, thence into the beakers as a very fine stream of bubbles. A control beaker was run simultaneously with test solutions. The control beaker contained only the nutrient (one-fourth strength Hoagland's medium) without the test drug. Preliminary experiments were conducted at room temperature. It was later found that the effect of colchicine is sensitive to temperature, but that of actidione is not to any appreciable extent. Subsequent experiments were performed in a constant temperature water bath maintained at 22.5±0.5°C. In so far as possible, all the data for this report have been taken from the temperature-controlled experiments.

The colchicine employed was a USP preparation of the Mallinkrodt Chemical Works bearing the control number BHG. Its structural formula (Muldoon 1950) has been determined to be:





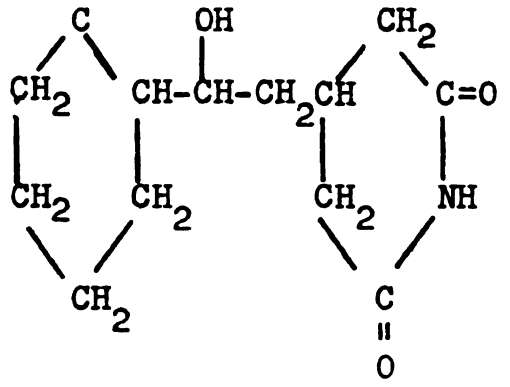
The colchicine was dissolved in distilled water to make a 0.1% or 1,000 parts per million stock solution. Table 1 relates percent, parts per million, and molar strengths for the drugs used. All dilutions used as test solutions were made from the above stock by buretting out the requied amount of stock solution into a beaker. The proper amount of Hoagland's nutrient was added to make a one quarter strength nutrient solution when brought up to 250 ml. total volume by addition of distilled water. The pH was a ascertained to be between 5.0 and 6.0 by the use of Hydrion paper. The dilutions of colchicine used were 30, 40, 50, 75 and 100 ppm. In all the experiments the treatment of the seedlings was continuous, and was allowed to progress for 24 hours. Seedlings remaining at the end of the treatment time were re-wrapped in wet paper toweling and allowed to recover. In all cases recovery was complete.

The prophase poison used in this investigation was the fungicidal antibiotic Actidione which is produced by Streptomyces griseus. It was obtained through the courtesy of the Upjohn Company of Kalamazoo, Michigan. The control number was FB-630. Actidione has a melting point of 113-115°C and a solubility in water of 25-30 mgm/milliliter at room temperature. It's structural formula is as follows:

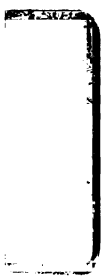
TABLE I

CONCENTRATION CONVERSION TABLE

MOLAR CONCENTRATION X 10^{-6}		PARTS PER MILLION	PERCENT
ACTIDIONE	COLCHICINE		
3550.0	2506.0	1000	0.1000
1775.0	1253.0	500	0.0500
355.0	250.6	100	0.0100
265.5	188.3	75	0.0075
177.0	125.5	50	0.0050
141.6	100.4	40	0.0040
106.2	75.3	30	0.0030
70.8	50.2	20	0.0020
35.4	25.1	10	0.0010
17.7	12.5	5	0.0005
3.6	2.5	1	0.0001



Actidione was dissolved in distilled water in sufficient amounts to make a 0.01% or 100 ppm stock solution. It was not made up in such large stock quantities as was the colchicine since it was used in smaller concentrations, and it was also found that actidione breaks down after an extensive storage period. In this investigation the unused stock was stored in the refrigerator for up to six months without undue effects. Dilutions were made in the same manner as for the colchicine. These dilutions were 1,5,10, 15 and 20 ppm. Treatment was continuous throughout the whole investigation. Actidione experiments did not have to be carried for so long a time as did some of the colchicine experiments since a cursory examination of the actidione treated tips after five to six hours showed so few division figures that analysis would have been impossible. Seedlings remaining at the end of the experiment were re-rolled in wet paper towels, which were inserted end down into a beaker, and the seedlings allowed to recover. Recovery was complete in all cases. However, the treated roots showed either much reduction or complete cessation of



primary root growth. The pH was ascertained to between 5.0 and 6.0 using hydrion paper. At no time was it necessary to adjust the pH with buffer solution in either the actidione or the colchicine experiments. The pH remained within the above limits throughout the experiment.

The sampling methods used in this investigation were similar to those used by previous workers of the Cytology group at Michigan State University (Bowen 1953, Hawthorne and Wilson 1952). As soon as the sprouted peas were unrolled and selected for treatment, at least three tips were immediately collected and fixed in three parts absolute alcohol: one part glacial acetic acid. This was termed the "Zero hour control." Once the experiment had started, samples were taken at least every hour and fixed as above. Not less than three tips were collected at each sampling point. In the case of one experiment samples were taken at less than one hour intervals. This exception will be noted as it arises in the observations.

The collected root tips were evacuated with a laboratory vacuum system for 15-20 minutes and then immersed in the fixative overnight. The present writer found that the root tips squashed better if fixed overnight, rather than for the shorter time which was used by previous investigators. This was especially so for the actidione treated tips, since the chemical appeared to make the root tips much more "woody".

The fixed root tips were hydrolysed 10-12 minutes in 1 N Hcl at 60°C. The HCL was then decanted and leucobasic fuchsin (Schiff's reagent) poured into the vial for staining. Here again, better results were obtained if the root tips were immersed in the stain overnight in the refrigerator, but in many cases the tips were squashed after an hour in the Feulgen reagent. The squashes were made by cutting off the reddened meristematic region of the root tip and placing it on a micrescope slide in a drop of a 0,1% Fast Green in 45% acetic acid solution. The tip was then thoroughly smeared with the flat end of a solid glass rod after which the whole mass was completely stirred to ensure as even a distribution of dividing cells as possible. A cover slip was then placed on the slide, which was gently heated over a low flame and the preparation was pressed firmly between paper towels. The slide was then dehydrated in 95% ethyl alcohol overnight. Following dehydration the slide was made permanent with diaphane.

OBSERVATIONS

There appears to be at least 4 cycles of events within the framework of mitosis which possibly could be upset by an outside agent (Wilson and Hyppio 1955). These 4 cycles are interdependent, but can be interrupted more or less independently to produce observable effects. They are:

(1) The movement cycle of the chromosomes.

(2) The spindle cycle.

(3) The morphological cycle of the chromosomes.

(4) The nuclear membrane cycle.

To these four cycles several others may be added and, of course, correlated cyto-chemical changes which must underlie all of the component cycles.

The movement and spindle cycles are apparently quite easy to interrupt, thus producing aberrant effects. It is difficult to produce changes in the morphological cycle, which appears to be at least semi-independent of the movement cycle.

Since this investigation is primarily concerned with anomalies produced in the movement cycle, a description of normal mitotic behavior with reference to movement of the

chromosomes will furnish a base from which to work.

In Pisum (Wilson and Hyppio 1955) the kinetochores of the seven pairs of chromosomes are polarized from the previous telophase. As early prophase is initiated the thin thread-like chromosomes appear as a tangled mass with the kinetochores grouped in a rosette at one pole. As prophase progresses the kinetochores start to move down the inside of the nuclear membrane towards the "equator." While this is going on the chromosomes are contracting. When the kinetochores reach a point about two thirds of the way from the pole to the equator, the nuclear membrane and the nucleoli apparently disappear. At this time the kinetochores begin a migration toward the center of the nucleus. This signifies that the end of prophase has been reached. When the kinetochores, with the chromosomes almost completely contracted, form a very loose group the configuration is termed prometaphase. From prometaphase the chromosomes migrate to the equatorial plate. This is the metaphase stage. Following metaphase, the kinetochores come apart and they, with their respective chromatids, start their journey toward the poles. This is anaphase. When the polar region is reached and the chromosomes start to uncoil, the configuration is termed telophase. Two restitution nuclei result, one on each side of a new cell wall formed along the equator of the original cell.

COLCHICINE

The configurations produced by colchicine are well known, and have been fully described by many previous workers in the field. In all the experiments of the present investigation the c-mitosis activity as described by Levan (1938) was achieved to a greater or lesser degree. It is upon the degree of aberration which this present investigation is, in large part, based.

The two major configurations observed in the colchicine treated seedlings are: (1) Scattered metaphases (Plate 1, Fig. 4 and 5) which if allowed to proceed would have become the typical c-mitotic configurations of Levan, and which suggest only partial effect on the spindle; (2) Clumped metaphases (Plate 1, Fig. 6) which if allowed to proceed would have gone through the morphological cycle in situ and become tetraploid. These clumps correspond to the "balled metaphases" of Bowen (1953) and Levan (1938, 1954). The presence of clumps suggests complete spindle destruction.

Examination of a preliminary experiment in which the doses were 30, 40, 50, 75 and 100 parts per million (ppm) suggested that the scatter configurations appear first with reference to time, and that the clumps appear later provided the dose was high enough to produce them. However, it must be emphasized that scatters do not give rise to clumps, since at low enough doses (in this case 30 ppm) scatters

PLATE I

Figures 1-9. Mitosis in Pisum sativum. Normal figures, and figures showing effect of colchicine and actidione.

Figure 1. Normal prophase, metaphase and telophase.

Figure 2. Normal late prophase, slightly distorted from the pressure of squashing.

Figure 3. Normal prometaphase.

Figure 4. Scattered configuration of colchicine. Treated with 30 PPM for three hours.

Figure 5. Same as Fig. 4, but slightly more "c-mitotic." Same dose and treatment time.

Figure 6. A typical colchicine clump. Treated with 75 PPM for three hours.

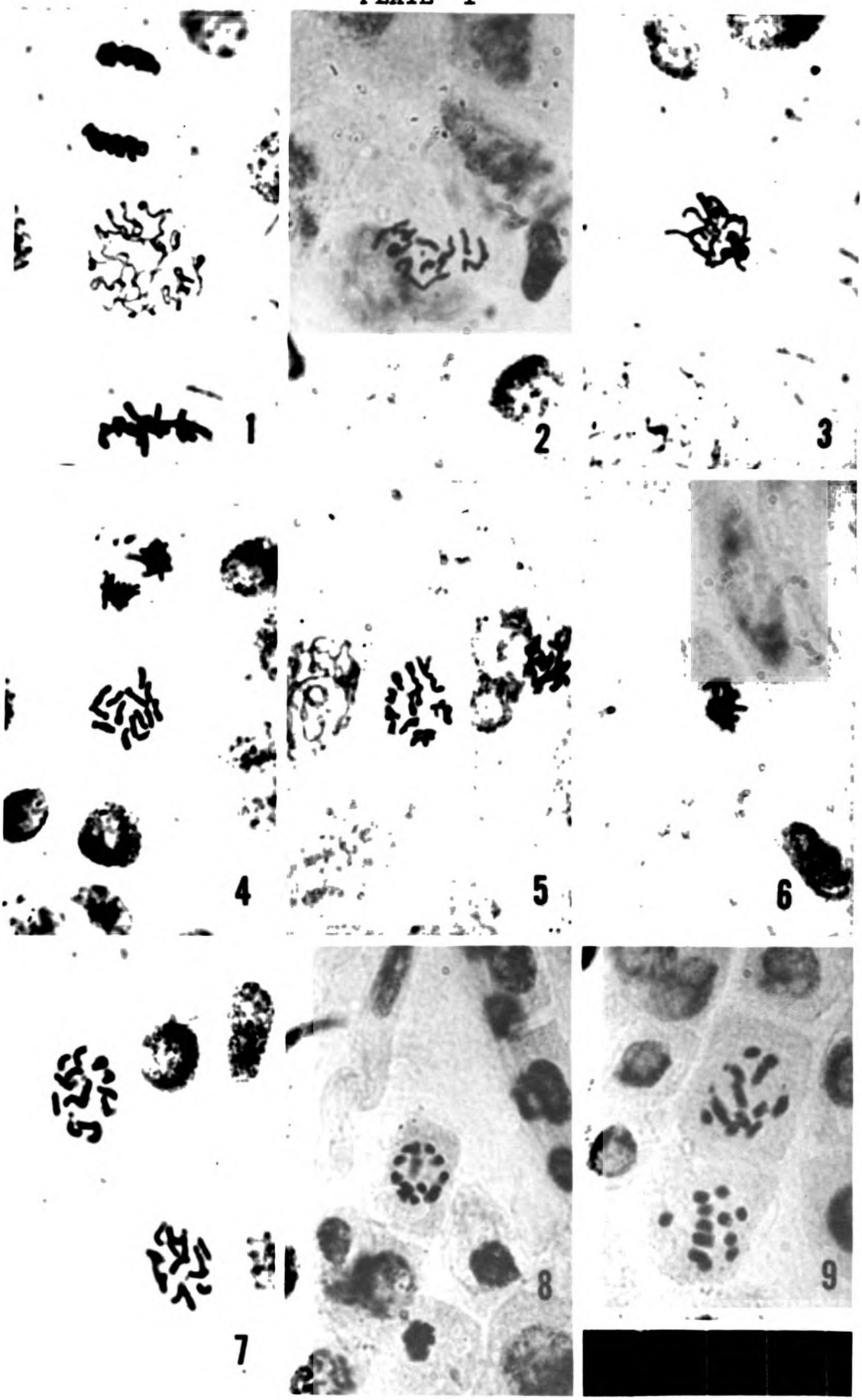
Figure 7. Actidione prophase, initial effect. Note the crescent shape of the chromosomes. Treated with 10 PPM for two hours.

Figure 8. Actidione prophase, more pronounced effect. Note the circular arrangement and the extreme contraction of the chromoeomes. Treated with 20 PPM for three hours.

Figure 9. Same as Fig. 8.

Each division of the scale represents 10 microns.

PLATE I



are the only colchicine effect noted; clumps never appear regardless of how long the seedlings are left exposed to the colchicine. This would suggest that a time-dose relationship exists for these effects.

The slides for each of the above doses were scored for frequency of scatters and clumps. These frequencies were lumped as "effect," and plotted as percent of effect against the total time during which the seedlings were subjected to the colchicine dose. The best estimate of the time when the frequency of scatters and clumps (lumped as effect) reached a measurable initial effect (10% aberrations), and when they reached "full" effect (90% aberrations) was made. These times are shown in Table II. When the times at which the various dose curves intersect the initial effect and full effect axes are plotted as the log of time versus the log of dose, a straight line is obtained.

This preliminary examination of seedlings treated with various doses of colchicine over a given period of time suggested that the cytological effects could be used on a quantitative basis to show a dose relationship through time. Since two configurations were recognizable, a method of distinquishing between them had to be made. Scatters appear before clumps, and since with low enough doses only scatters are attained, it was assumed that scatters represented a less severe cytological effect than did clumps. On the basis of this assumption the following arbitrary values were

TABLE II

BEST ESTIMATE OF TIME THAT SEEDLINGS REACHED INITIAL AND FULL EFFECT FOR VARIOUS DOSES OF COLCHICINE

DOSE	TIME IN MINUTES	
PPM	INITIAL EFFECT	FULL EFFECT
30	1260*	----
40	660	1260
50	480	780
75	150	300
100	60	120

* Uncertain

assigned:

Configuration	Symbol	Weight
Normal	X	0
Scattered	Y	1
Clumped	Z	2

The number of cells on a slide showing a scattered configuration was multiplied by one, and that showing clumps was multiplied by two. The two resulting values were added and the total divided by the total number of post prophases counted which was 200 in all cases with the exception of a few slides which did not have 200 post-prophases on them. The resulting values have been termed "Indices of Effect" (I.E.). This may be expressed simply as follows:

$$\text{I.E.} = \frac{1(Y) \quad 2(Z)}{n}$$

All three slides from each sampling point were treated in this manner and an average of the indices was taken.

The experiment using 30, 40, 50, 75 and 100 ppm was scored for index of effect with the results listed in Table III. All the points needed to show a full spread of effect were not determined, thus it is not known precisely when full effect was reached with reference to the index. It is to be noted that the 40 ppm reaches only an index of a little over 1.00 in 24 hours, while the 30 ppm concentration turned out to be so near the threshold dose, that only an index of 0.2 was achieved (See foot-note, Table V). This experiment furnished the preliminary information on which to base more

TABLE III

INDEX OF EFFECT FOR 40, 50, 75 AND 100 PPM COLCHICINE

TIME IN HOURS	INDEX OF EFFECT			
	40 PPM	50 PPM	75 PPM	100 PPM
1	-	-	0.06	0.09
2	-	-	0.32	1.00
3	-	-	0.46	1.40
4	-	-	0.81	1.57
5	-	-	1.01	-
6	-	0.24	1.46	-
7	-	0.47	-	-
8	-	0.28	-	-
9	-	0.57	-	-
10	-	1.01	-	-
11	-	0.88	-	-
12	0.21	0.87	-	-
13	0.19	1.07	-	-
14	0.22	-	-	-
15	0.38	-	-	-
16	0.34	-	-	-
17	0.47	-	-	-
18	0.23	-	-	-
19	0.51	-	-	-
20	0.52	-	-	-
21	0.88	-	-	-
22	-	-	-	-
23	0.85	-	-	-
24	1.13	-	-	-

extensive investigations.

On the basis of the above information an experiment was set up using 40 and 50 ppm. Samples were taken at various intervals and for a sufficient time to ensure that the whole range of effect had been covered. Upon scoring, it was found that both doses reached an index of effect close to 2.0 (Table IV). It must be pointed out that even the most massive doses will not result in an index of 2.0 since there will always be some normal figures. The plot of these indices against time yields curves with sigmoidal tendencies (Text figs. 1 and 2). As expected, the 50 ppm reaches its full effect about 3/4 of an hour before the 40 ppm.

In order to test the validity of the sigmoid curve achieved with these two doses, the indices were subjected to a probit analysis. According to Finney (1952) probit analysis will turn a true sigmoid curve into a straight line. It is to be noted that these sigmoid curves do indeed meet the straight line requirements on probit analysis (Text figs. 3 and 4), the slopes for which have been ascertained to be 0.42 for the 40 ppm, and 0.32 for the 50 ppm (Appendix I).

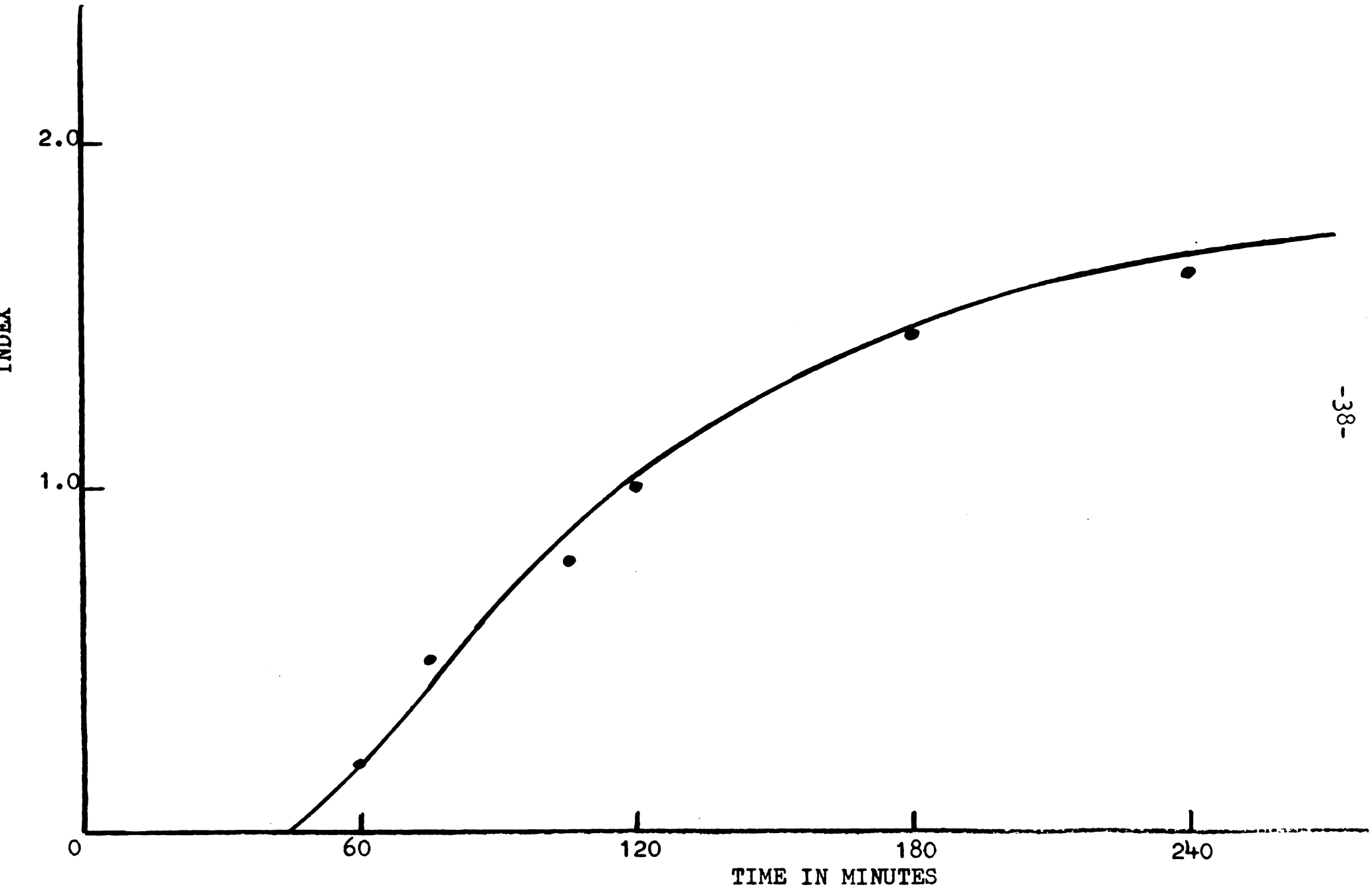
It should be noted that the rate of change of the index in the 40 and 50 ppm experiment is much greater than in the original experiment. This difference apparently reflects a temperature factor which was uncontrolled in the first experiment, but averaged several degrees lower.

Upon determining that the change in index through time

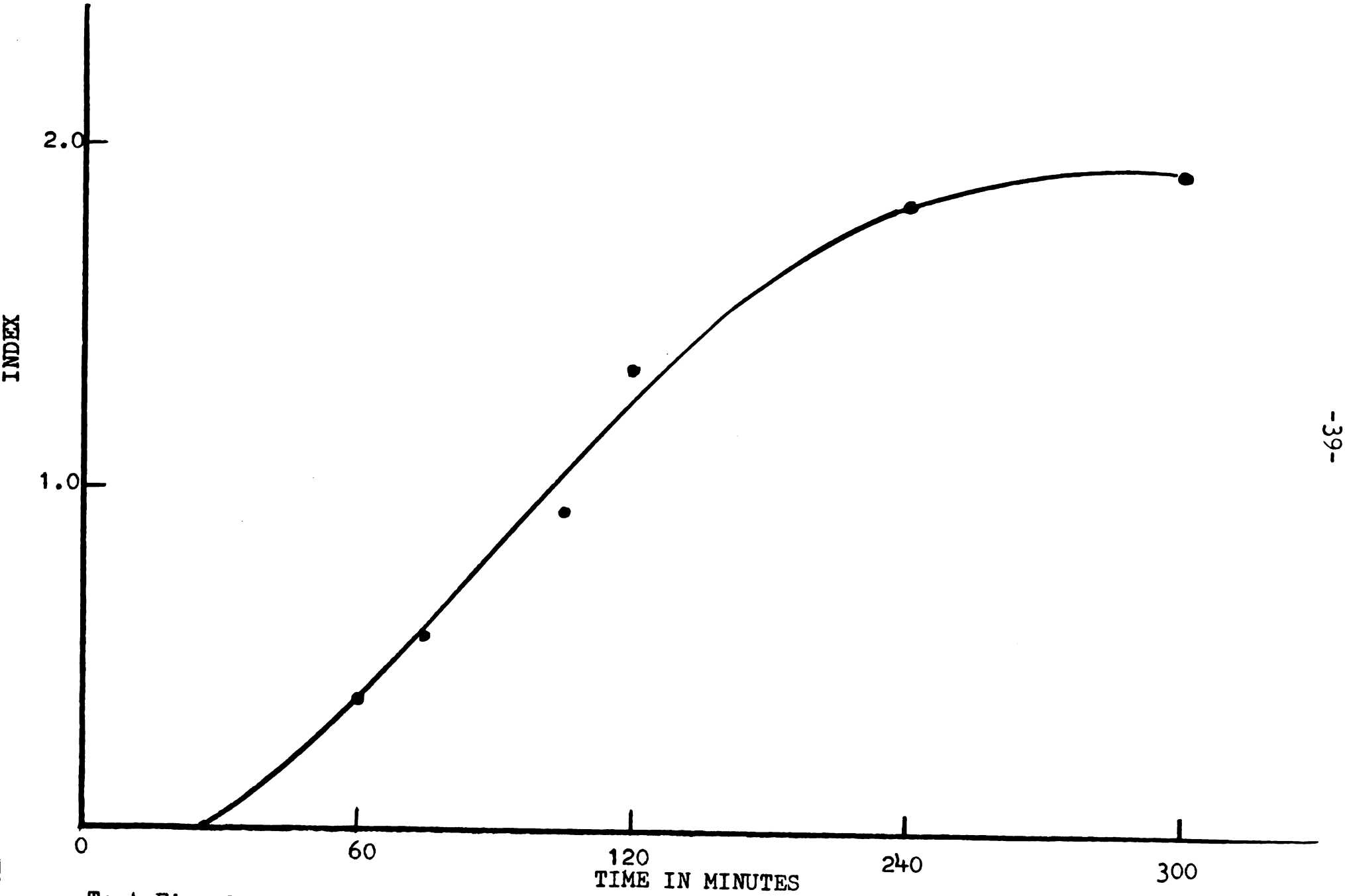
TABLE IV

INDEX OF EFFECT FOR 40 AND 50 PPM COLCHICINE

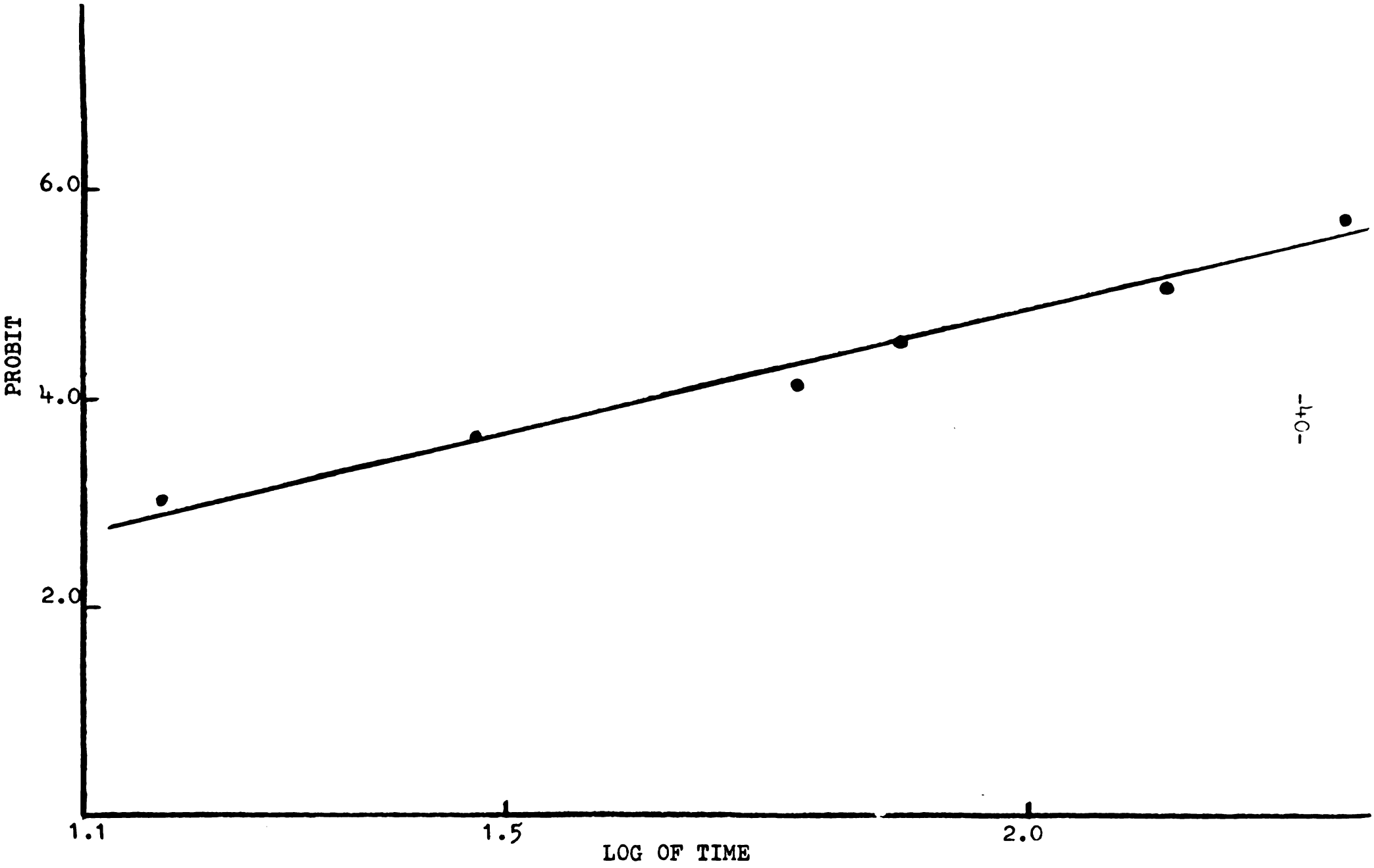
TIME IN HOURS	INDEX OF EFFECT 40 PPM	50 PPM
1	0.20	0.39
1¼	-	0.57
2	1.00	1.35
3	1.44	1.84
4	1.83	1.93
5	1.92	-



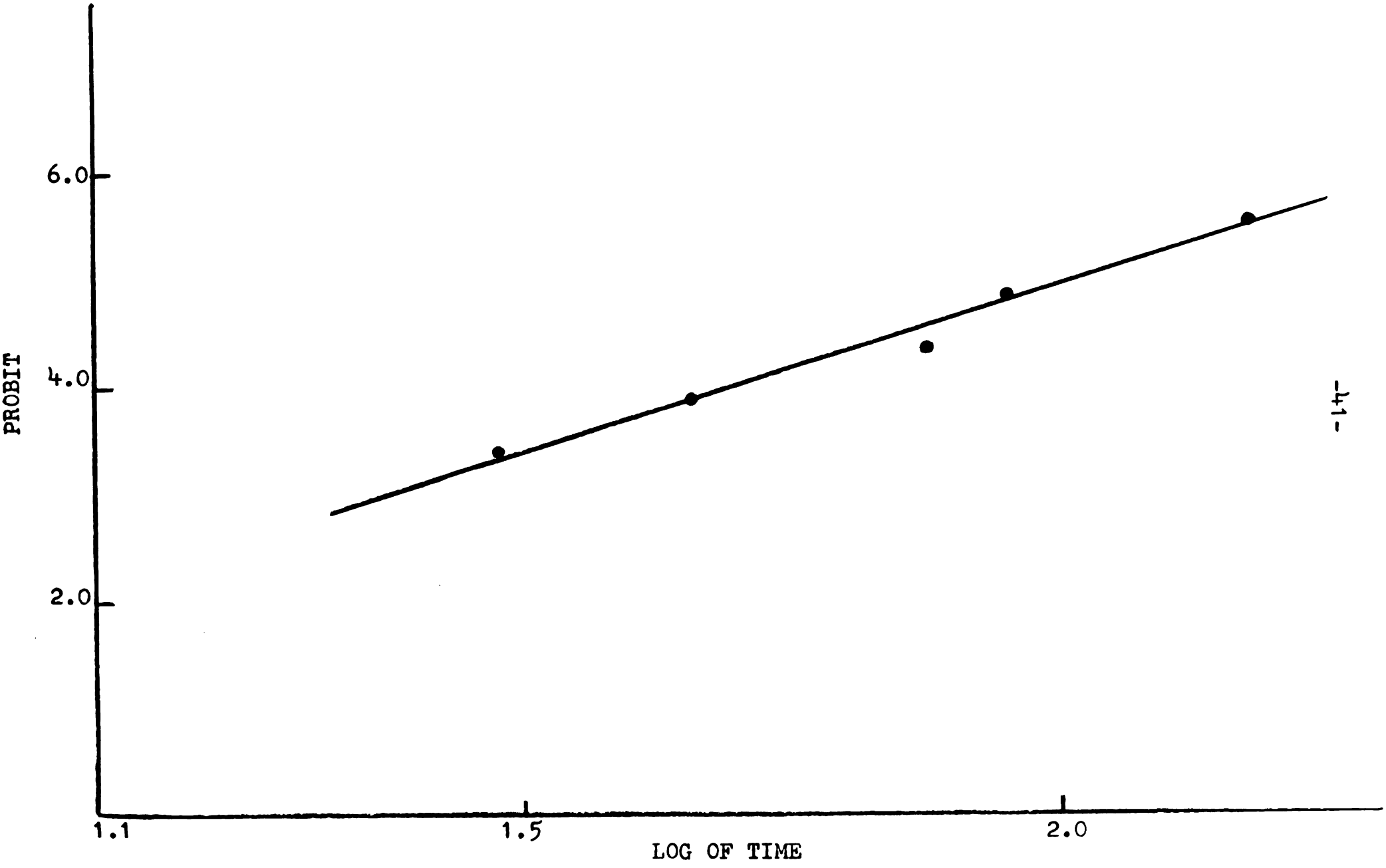
Text Fig. 1. Colchicine: Change in index of effect through time (40 PPM).



Text Fig. 2. Colchicine: Change in the index of effect through time (50 PPM).



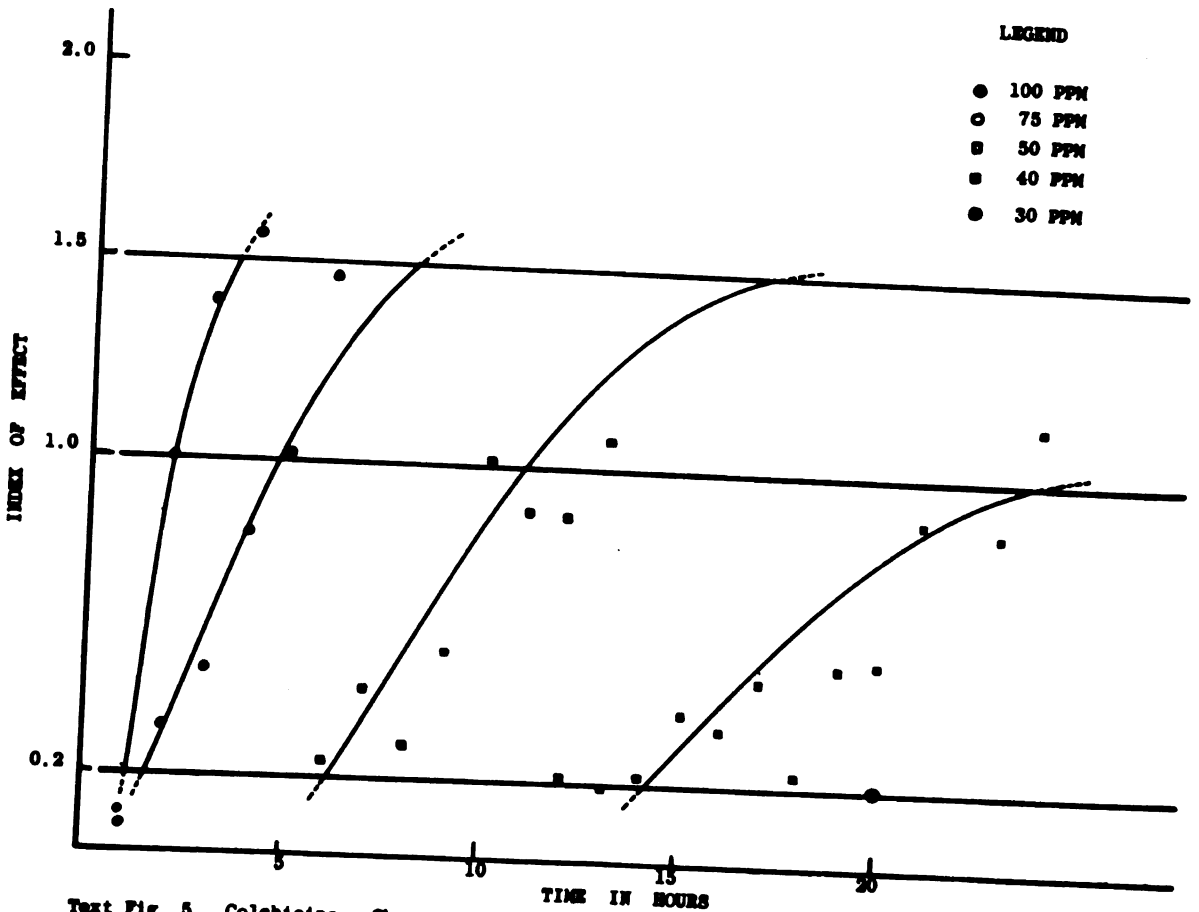
Text Fig. 3. Colchicine: Probit analysis line (40 PPM).



Text Figure 4. Colchicine: Probit analysis line (50 PPM).

for various doses of colchicine follows a sigmoid curve, the first experiment using 30, 40, 50, 75 and 100 ppm was re-examined. On the basis of the results obtained in the 40 and 50 ppm experiment, all available indices of the first experiment were plotted and fitted with the appropriate sigmoid curve (Text fig. 5). It is to be noticed that the material exposed to 40 ppm was very erratic in the distribution of these indices, although an index of 1.13 was reached in 24 hours. This is typical of concentrations that are at or near the threshold dose. The 50 ppm shows a decidedly better progression towards full effect, and reaches an index of 1.07 in 13 hours. It would reach an estimated index of 1.5 in about 17 hours on the basis of the extension of the sigmoid curve. The 75 ppm concentration produces an index of close to 1.5 in 6 hours, and the 100 ppm produces an index of slightly over 1.5 in four hours. All the above noted times are in terms of time after the initiation of treatment.

Intercepts were arbitrarily fixed at 0.2, 1.0 and 1.5 on the index scale (Text fig. 5). Intercepts above 1.5 were not used due to the difficulty of making precise calculations above this point. Table V lists the times at which the various dose curves pass through the intercepts. Since the preliminary experiments gave a straight line on a log/log plot for time-dose at initial and full effect times, it would be reasonable to expect that if the times at which the



Text Fig. 5. Colchicine: Change in the Index of Effect with time.

TABLE XII

BEST ESTIMATE OF THE TIME AT WHICH THE DOSE CURVES CROSS THE VARIOUS INTERCEPTS (E/L)

DOSE PPM	INTERCEPTS IN MINUTES 1	2	3
5	211	174	142
10	153	108	78
15	139	100	70
20	129	93	68

of 7.6. It can be seen that the lower doses take about an hour to stabilize themselves. Once the curves have stabilized the ratios show a constant decrease toward 0 with time. It will be noticed that in the 1 ppm treatment the ratio does not reach 0, but rather a new equilibrium, and levels off because the inhibition of new mitoses is not complete, and because some potential metaphases are going through the actidione pathway (See discussion).

Here again, since there appears to be a steady decrease in the ratio with time, intercepts may be arbitrarily chosen in the stabilized regions of the curves. Those used are 1.0, 2.0 and 3.0 (Text fig.9; Table XII). If the times at which the dose curves intersect the intercepts are plotted on a log of time versus a log of dose basis for each intercept, they are found to give straight lines whose slopes do not differ significantly (Text fig. 10C) (Appendix II).

It also can be noted that the slopes of this third set of measurements do not differ significantly from the slopes obtained in the two previous types of measurements.

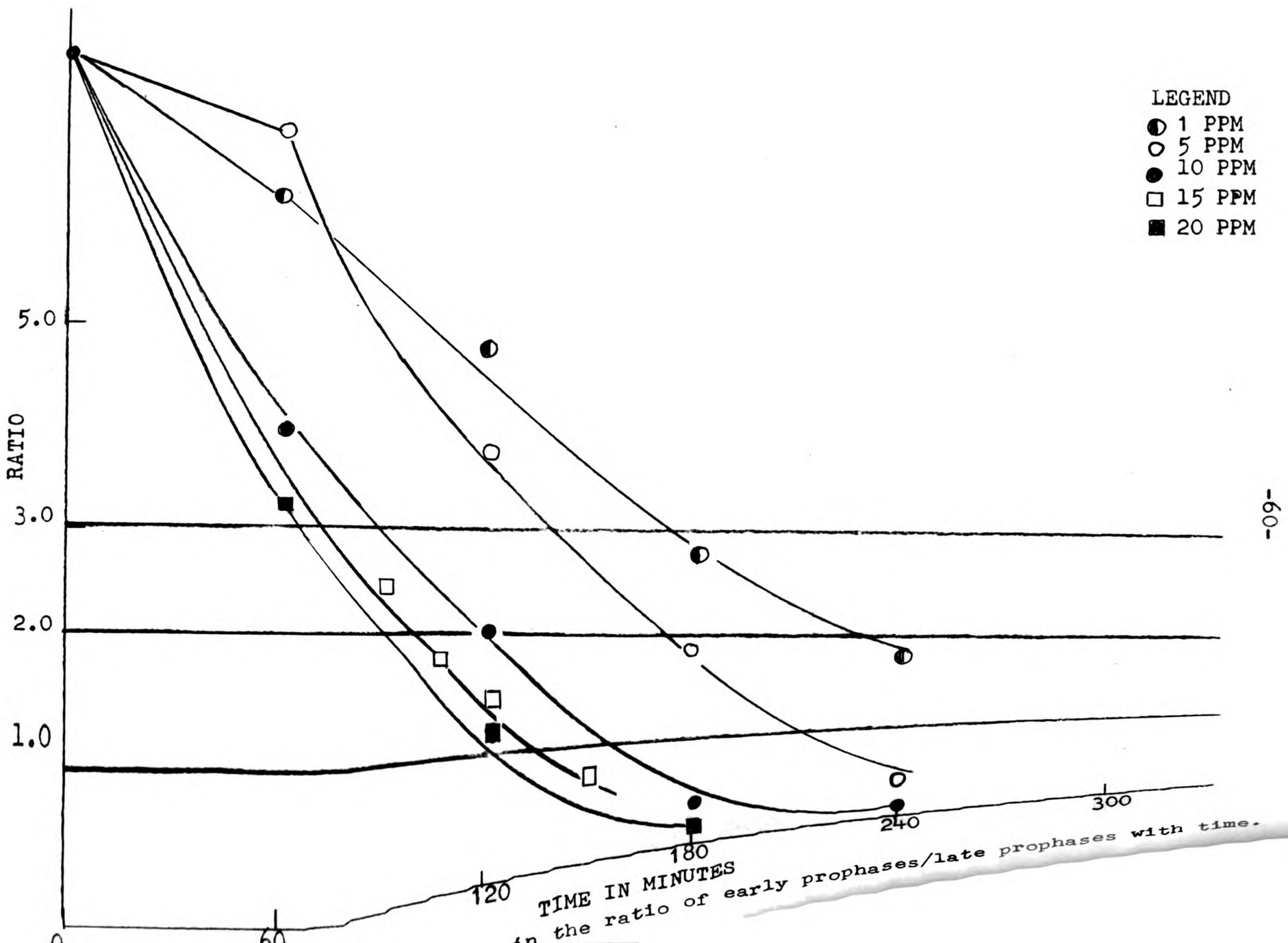
The achievement of straight and parallel lines for this third measurement would seem to indicate that it is a valid combination of the two previous measurements.

A statistical analysis shows that the lines obtained in all three types of measurements do not differ significantly in slope, and that a common straight line may be drawn for all three (Text fig. 10D). Thus they may be presumed to

TABLE XI

CHANGE THROUGH TIME IN THE RATIO OF EARLY PROPHASES TO LATE PROPHASES

TIME IN MINUTES	RATIO 1 PPM	5 PPM	10 PPM	15 PPM	20 PPM
0	7.60	7.60	7.60	7.60	7.60
60	6.20	6.80	4.00	-	3.50
90	-	-	-	2.50	-
105	-	-	-	1.67	-
120	4.80	3.80	2.00	1.48	1.20
150	-	-	-	0.76	-
180	2.80	1.90	0.38	-	0.21
240	1.80	0.47	0.10	-	0.20
420	2.20	-	-	-	-



in the ratio of early prophases/late prophases with time.

significantly (Appendix II). It is to be noted likewise, that the slopes obtained for this measurement do not differ significantly from the sloped obtained in the measurement of actidione prophases expressed as a percent of normal late prophases (Appendix II). This would indicate that these two types of cytological measurements measure the same thing.

If the two previously recorded types of measurements are valid, the first of which is based on a cytological configuration, and the second of which is based on mitotic inhibition, then a combination of these two types of measurements should also be valid, and should show the same type of straight line relationship. If the initiation of mitosis is increasingly inhibited with time when the meristematic tissue is treated with actidione, then the ratio of early prophases to late prophases (E/L), regardless of whether or not they show cytological anomalies associated with actidio should steadily progress toward 0 at a constant rate for a given dose. It must be remembered, however, that the progression of this E/L ratio toward 0 will be accelerated i some of the late prophases are stalled and go through th remainder of the morphological cycle <u>in situ</u> (Via the actidione pathway, Text fig. 11). These will then be s spatially as late prophase.

Upon scoring all doses for an E/L ratio, results w obtained as in Text fig. 9; Table XI. The starting poi all the curves is the "zero hour" control with an E/L r

TABLE X

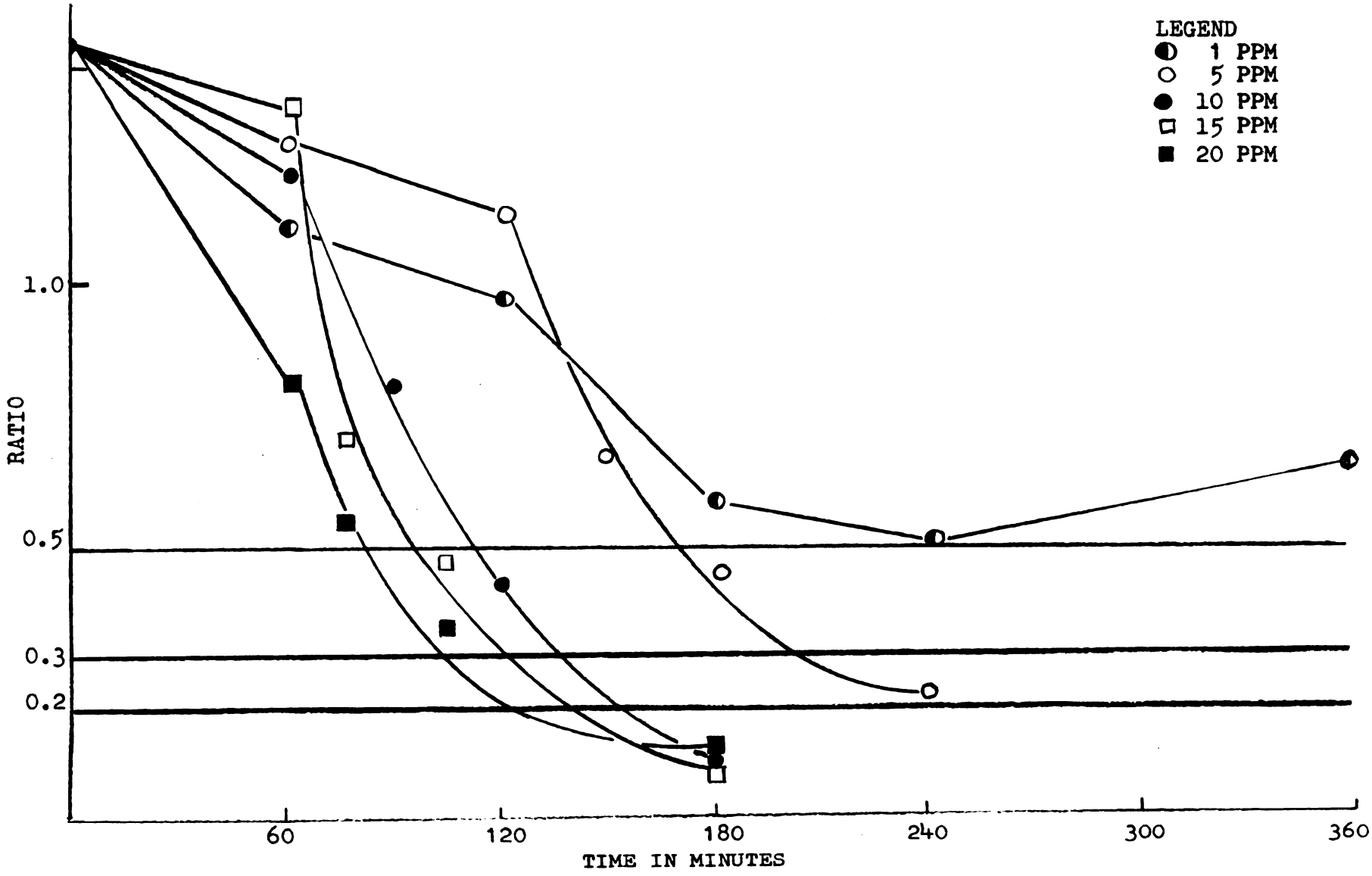
BEST ESTIMATE OF THE TIME AT WHICH THE DOSE CURVES (EARLY PROPHASE/TOTAL REMAINING FIGURES) CROSS THE VARIOUS INTERCEPTS

DOSE PPM	INTERCEPTS IN MINUTES 0.2	0.3	0.5
5	240	202	170
10	159	142	115
15	140	120	95
20	116	101	81

TABLE IX

CHANGE THROUGH TIME IN THE RATIO OF EARLY PROPHASES TO THE TOTAL OF ALL REMAINING MITOTIC FIGURES

TIME IN MINUTES	RATIO 1 PPM	5 PPM	10 PPM	15 PPM	20 PPM
0	1.44	1.44	1.44	1.44	1.44
60	1.10	1.26	1.20	1.33	0.81
75	-	-	-	0.71	0.55
90	-	-	0.81	-	-
105	-	-	-	0.48	0.35
120	0.94	1.13	0.43	0.30	0.13
150	-	0.67	-	-	-
180	0.60	0.46	0.09	-	-
240	0.52	0.23	-	-	-
360	0.63	-	-	-	-



Text Fig. 8. Actidione: Change in the ratio of early prophases/total of all remaining mitotic figures with time.

accurately; the second because it would involve a prohibitive number of measurements. Therefore a measurement was used such that a time at which a given ratio was attained was plotted against dose. If the rate of change is regular, then any arbitrarily chosen ratio would show the same time-dose relationship, and would also show the same relationship that would be expected if the theoretical measurements expressed in (1) and (2) above were used.

Upon scoring all sampling points for all doses it was found that the index obtained went down with time. Except for the 1 ppm treatment, in which the ratio simply fluctuated, the downward trend was regular (Text fig. 8; Table IX).

Since a glance at Text fig. 8 would indicate that there is a constant rate of fall of the indices for all the doses except the 1 ppm, then intercepts can be chosen which should indicate the same theoretical relationship as would the starting points of mitotic inhibition for the various doses. It is to be noted that these intercepts were chosen such that they lie in the stabilized regions of the curves. No intercept above 0.6 was used since this would be coming too close to the times when the curves fluctuate during the first hour of treatment. The time when the dose curves cross the intercepts (Table X) has been plotted on a log of time versus a log of dose basis (Text fig. 10B). It will be noted that each intercept plot gives a straight line, the three lines are parallel, and the slopes do not vary

exposures regardless of whether time or dose is used as the variable.

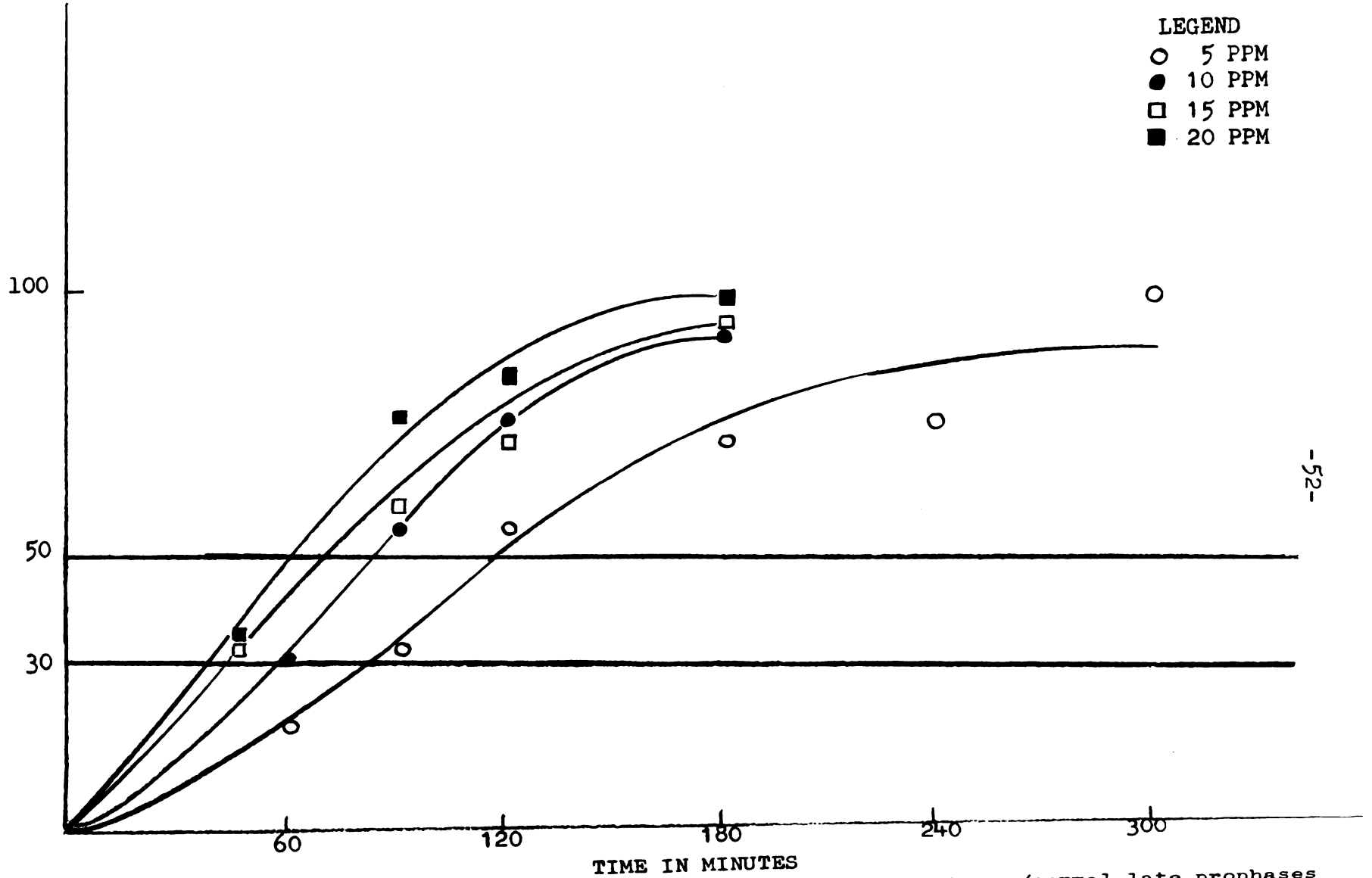
It was previously mentioned that the mitotic index went down with time in the actidione treated material (Table VI). It may also be noted that this index went down more rapidly the higher the dose up to a certain dose. After the lethal dose was reached (probably 30 ppm and above) the mitotic index appeared not to go down at all (See also Hawthorne 1951). This is actually a false effect as such a dose is strong enough to "fix" the cells, thus everything remains in the stage it was in when the treatment was initiated. Although the general trend was for the mitotic index to decrease with time in the treatment, it was found that to use the mitotic index as a direct measure of cytological effect was not feasible since the fluctuations were too great, even within a single sampling point. However, this depression of mitotic index did suggest that a related measure might be used. It was discovered that if the number of early prophases was expressed as a proportion of the total of the remaining division figures, a fairly uniform change occurred. Such a change can be used as a measure of mitotic inhibition.

Theoretically there are two times at which the frequency of early prophases versus the total of all remaining figures is equal to 0. Neither of these theoretical times is feasible. The first because it cannot be predicted

TABLE VIII

BEST ESTIMATE OF THE TIMES AT WHICH THE DOSE CURVES (% ACTIDIONE PROPHASES) CROSS THE VARIOUS INTERCEPTS

DOSE PPM	INTERCEPTS IN MINUTES 30 %	50 %
5	94	118
10	63	84
15	45	70
20	40	57



Text Fig. 7. Actidione: Change in the percent of actidione prophases/normal late prophases through time.

TABLE VII

CHANGE THROUGH TIME IN THE NUMBER OF ACTIDIONE PROPHASES EXPRESSED AS PERCENT OF NORMAL LATE PROPHASES

TIME IN MINUTES	PERCENT			
	5 PPM	10 PPM	15 PPM	20 PPM
0	0	0	0	0
45	-	-	34	35
60	19	31	-	-
90	33	55	60	77
120	56	76	73	84
180	72	91	94	98
240	75	-	-	-
300	98	-	-	-

Since actidione prophase configurations appear to be late prophase spatially, it would seem reasonable to contend that if the number of actidione prophases was related to the number of normal late prophases, a measure of cytological effect would have been achieved. The number of actidione prophases for each dose at each sampling point was scored, averaged, and tabulated as percent of normal late prophases (Table VII). Upon plotting these percentages against time a sigmoidal type curve is obtained (Text fig.7). Each dose reached maximum effect with the exception of the 1 ppm. Since this concentration was so erratic when scored, it was discarded as a valid, measurable dose for this particular type of measurement. As would be expected, the higher the dose, the sooner maximum effect was reached. Thus it is seen that the 20 ppm dose reached a value of 98% in 3 hours; the 15 ppm reached a value of 94% in 3 hours; the 10 ppm a value of 91% in 3 hours; the 5 ppm a value of 98% in 5 hours.

Arbitrary intercepts were chosen at two places where it was obvious that the curves had stabilized. These are the 30% and 50% intercepts. The times at which these dose curves cross the intercepts when plotted in a log of time versus a log of dose basis give a straight line (Text fig. 10A; Table VIII), the slopes of which are not significantly different (Appendix II). This straight line relationship would appear to indicate a smooth progression of events, and that the same mechanism is affected at different

TABLE VI

CHANGE IN MITOTIC INDEX THROUGH TIME FOR VARIOUS DOSES OF ACTIDIONE

TIME IN MINUTES	CONCENTRATION IN PPM				
	1	5	10	15	20
0	48	48	48	48	48
60	39	47	49	29	36
120	53	40	29	25	33
180	34	34	27	17	25
240	28	15	22	16	16
300	21	-	-	-	-
420	21	-	-	-	-

verge of becoming telomorphic, which indeed they do as time goes on. Very pronounced actidione prophases show the chromosomes as short arcs compressed against what appears to be a restraining structure which may be the nuclear membrane. Plate 1, Figs. 7, 8 and 9 show some typical actidione prophases.

Experiments were performed using 1, 5, 10, 15 and 20 ppm (See Table I for the relationship between parts per million, percent, and molar concentrations). All the experiments were done in a temperature controlled water bath at $22.5 \pm 0.5^{\circ}C$, and all the sampling conditions were the same as for colchicine.

A cursory examination of the various dosage effects established the fact that the actidione prophases became more conspicuous with time, and that they appeared earlier, the higher the dose. This, of course, immediately suggested a time-dose relationship. It was also at once apparent that there was an effect on the initiation of mitosis, since after four or five hours all division figures had disappeared from the treated material, while the control material remained at a fairly constant division level. A complete check on the mitotic index for all three slides at each sampling point confirmed the inhibition effect. This decrease in mitotic index with time (Table VI) will be used later as a basis for establishing a ratio by which the inhibition effect can be studied.

(2) All of these straight lines have a common slope.

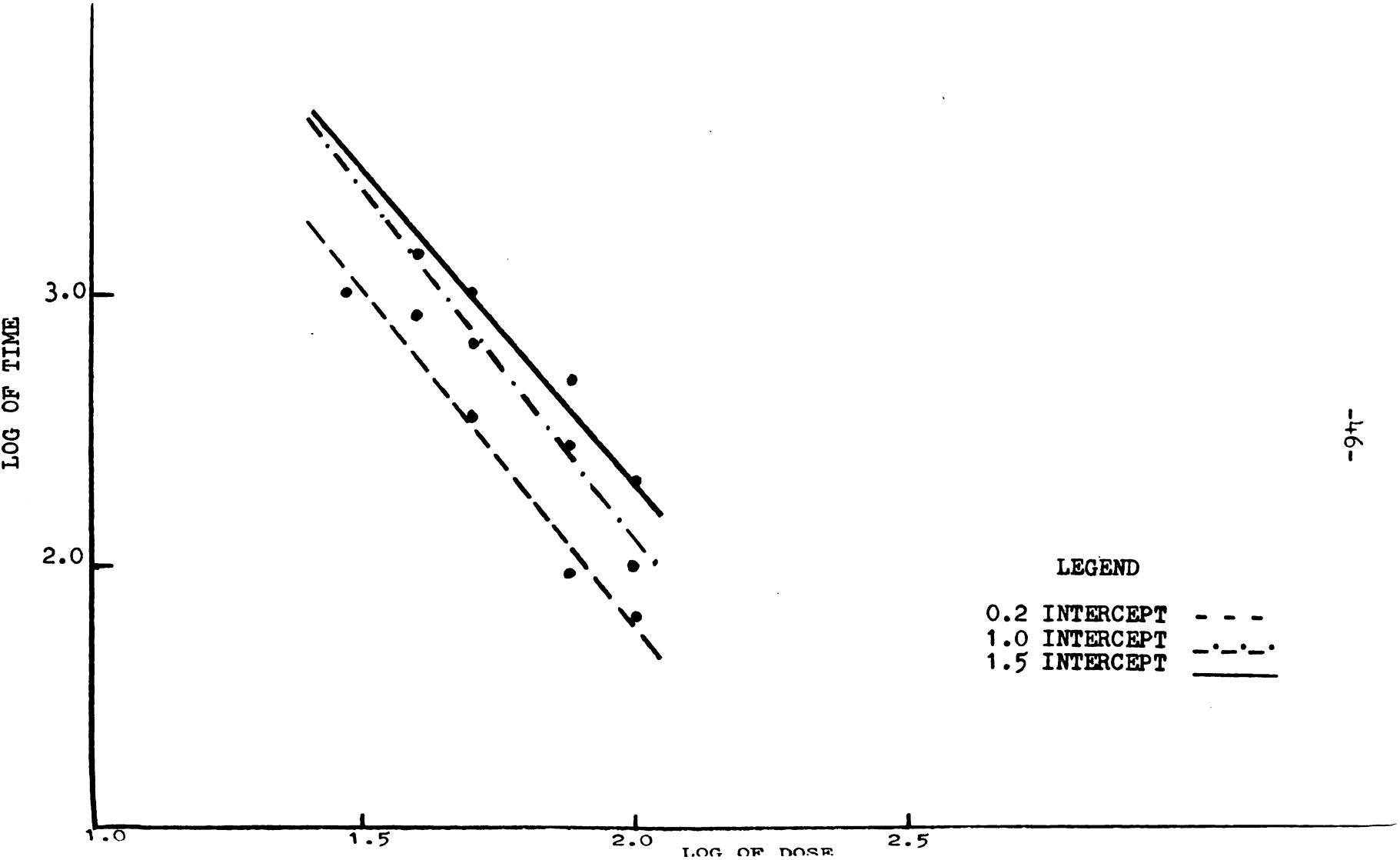
(3) The kind of effect does not change with either time or dose.

(4) The effect must take place sometime between late prophase and metaphase.

ACTIDIONE

This drug was first reported by Whiffen, Bohones and Emerson (1947). The first comprehensive study of its effects on meristematic tissue was undertaken by Hawthorne and Wilson (1952) and by Bowen and Wilson (1954). These investigations showed that actidione tends to inhibit the onset of mitosis, and likewise has an effect on the prophase configurations that are already in the mitotic cycle.

Preliminary investigations in the present study completely confirmed the above results. As in the colchicine studies it is necessary here to establish the presence of one or more recognizable, measurable cytological anomalies. Two such appear potentially usable as measures of effect. These are: (1) a configuration which hereinafter is referred to as an actidione prophase, and (2) the obvious depression of mitotic index. Spatially an actidione prophase configuration is a late prophase. The chromosomes appear quite contracted, more so, in fact, than usual at late prophase. Also they appear fuzzy and look as if they might be on the


LOG OF TIME
3.0
2.0
1.0
1.5
2.0
2.5
LOG OF DOSE
LEGEND
0.2 INTERCEPT
1.0 INTERCEPT
1.5 INTERCEPT

index change passed through the various intercepts were also plotted on the same basis, they would give a straight line. Text fig. 6 illustrates that a straight line is indeed achieved for the intercepts 0.2, 1.0 and 1.5. It can also be observed that all three lines are parallel. The slopes for each of these has been calculated to be:

Intercept 0.2: -2.6433
Intercept 1.0: -2.6065 (See appendix I for
Intercept 1.5: -2.2232 calculations)

These slopes were found not be be statistically different.

In none of the experiments was it found that colchicine depressed the mitotic index or had any toxic effects on the cells in the concentrations used. The scattered configurations eventually became multinucleated cells and the clumps eventually became tetraploid nuclei. In all the experiments the seedlings that were left after the experiment was finished were re-wrapped in wet paper towels. Recovery was 100%.

SUMMARY

In summarizing the colchicine observations, the following points can be listed:

(1) A linear relationship exists between time and dose as measured by cytological effect when plotted on a log of time/log of dose basis.

TABLE V

BEST ESTIMATE OF THE TIME IN MINUTES AT WHICH VARIOUS DOSE CURVES PASS THROUGH THE INTERCEPTS

DOSE PPM	TIME IN MINUTES INTERCEPTS		
	0.2	1.0	1.5
30*	1200	-	-
40	860	1440	-
50	372	654	1020
75	96	282	480
100	66	120	212

* The 30 PPM dose is so close to the apparent threshold concentration that only the 0.2 intercept was measurable.

Text Fig. 10. Actidione: Linear relationship of all types of measurements.

(A) Actidione prophases as % of normal late prophases

30% ————————
50% ----------

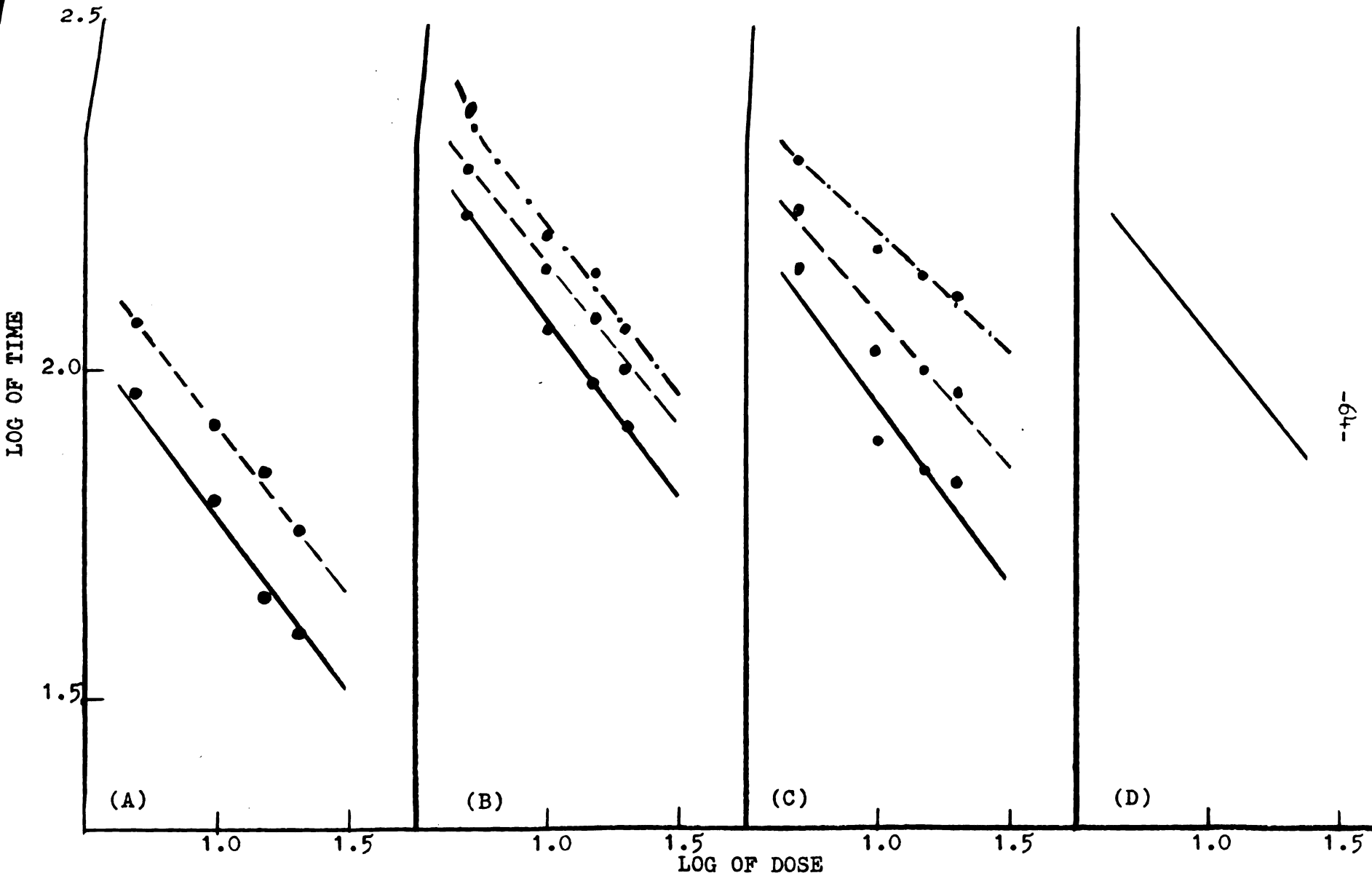
(B) Early prophase as a ratio of total of remaining mitotic figures

Intercept 2: ·-·-·-·-·-
Intercept 3: ---------
Intercept 5: ————————

(C) Early prophases as a ratio of late prophases

Intercept 1: ·-·-·-·-·-
Intercept 2: ---------
Intercept 3: ————————

(D) Common slope for all types of measurements



Text Fig. 10. Actidione: Linear relationship of all types of measurements.

measure the same thing.

SUMMARY

The actidione observations may be summarized as follows:

(1) Three types of measurements are used to determine events and relationships.
   a. Configuration based measurement.
   b. Ratio based measurement.
   c. A measurement combining the above two.

(2) All measurements give a series of straight and parallel lines.

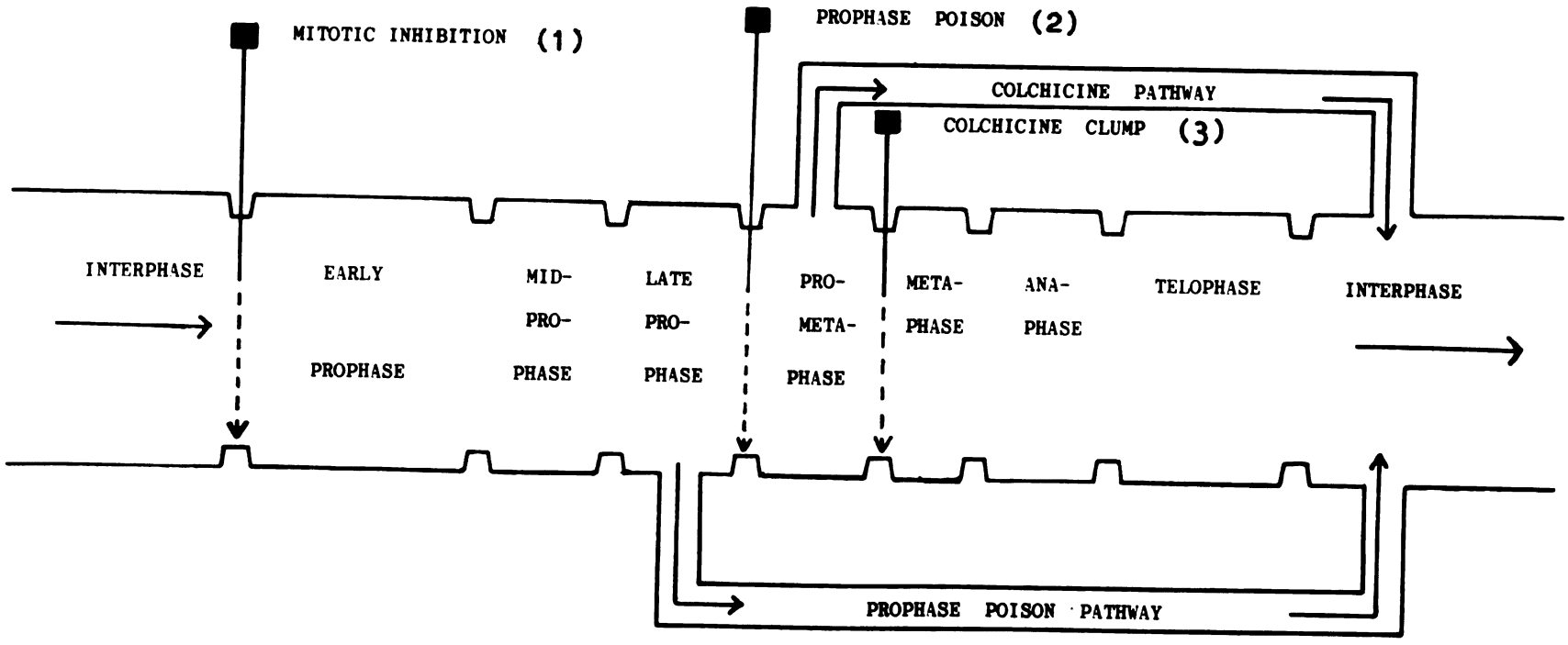
(3) All measurements can be reduced to a single straight line within their respective measurement type.

(4) A single straight line representing events and relationships between the three measurement types can be derived.

(5) The time-dose relationship is linear on a log/log basis.

DISCUSSION

In an effort to better portray the effects which the drugs used in this investigation have on the movement cycle of mitosis, a diagrammatic sketch has been drawn up (Text fig. 11). This diagram illustrates the progress of chromosome movement through the normal cycle, the colchicine cycle, and the actidione cycle. The normal chain of events is represented by progression straight through the diagram starting at interphase on the left, and going to the next interphase on the right. This straight-forward progression is divided off into the various arbitrary stages of mitosis. On the other hand, aberrant mitosis under the effect of the drugs used here is represented by certain deviations or detours from the normal straight-line progression. The action of the drugs is represented by "valves" at the points at which they are postulated to work. Thus, in the case of colchicine, if the "valve" (3) just prior to metaphase is pushed down (representing colchicine effect), normal chromosome movement cannot progress into metaphase, but rather is forced through the detour marked "Colchicine pathway." This detour leads to the next interphase on the



Text Fig. 11. DIAGRAM REPRESENTING INTERFERENCE WITH MITOTIC MOVEMENT CYCLE IN PISUM

right, thus showing that colchicine effected configurations while in the detour pathway are undergoing morphological changes *in situ*. This detour results in either tetraploidy or multinucleated cells, depending on the degree of colchicine effect.

While colchicine effect can be illustrated by the use of one "valve," actidione effect must be illustrated by two "valves." Actidione is regarded as a prophase poison as defined by D'Amato (1949), and its prophase poison effects have been established by Hawthorne (1951) and Hawthorne and Wilson (1952). As a prophase poison, actidione, besides affecting prophase stages of mitosis, also effects the initiation of new mitotic configurations. This is very evident from the results of the present investigation (Table 6, Table 9, and Text fig. 8). Consequently if the first "valve" (1) is pushed down (representing the inhibitory phase of actidione action) no new mitoses will enter the cycle. It will be noted in a subsequent section of the discussion that the first and second "valves" are not always pushed down all the way. On the other hand, if the second "valve" (2) (situated between late prophase and prometaphase) is pushed down (representing the prophase poison phase of actidione action), the movement cycle of the chromosomes progresses to late prophase spatially, then the morphological cycle is continued *in situ*, this being represented by the actidione detour pathway.

COLCHICINE

There is no doubt whatsoever that the generally held concept of the action of colchicine on the mitotic cycle resides in its ability to effect in some way or another the formation and action of the spindle mechanism. This blocking of the spindle function results in polyploidy and other nuclear anomalies such as multinucleated cells and microcytes. The production of the so-called "clumps" in meristematic tissue treated with colchicine is a result of the complete destruction of the spindle mechanism or its precursor such that the chromosomes, once they have reached prometaphase, are unable to go any further. It would appear that the presence of the spindle structure is necessary for the chromosomes to move from the prometaphase configuration (Plate 1, Fig. 3) out to the metaphase plate and their consequent orientation.

This destruction of the spindle causes the chromosomes to continue their morphological cycle <u>in situ</u>; the chromatids fall apart after considerable stalling by the action of the drug. Since there is no spindle to produce pole-ward migration, the chromosomes become telomorphic and eventually reconstitute an interphase nucleus which is tetraploid.

The presence of abnormal telophase and interphase configurations, other than polyploid ones, has been regarded as a sign of partial spindle destruction. This may have

taken place during the treatment or later, during recovery. In the present investigation it is suggested that the cytological configurations scored as "scatters" resulted from only partial spindle destruction; there was, perhaps, enough of the spindle left to partially organize a metaphase configuration, thus the chromosomes are in a sort of "helter-skelter" arrangement on the metaphase plate. The ultimate in scattering is, of course, the typical c-metaphase of Levan (1938,1940,1951,1954). It would appear that the majority of scatters result in multinucleates and microcytes, rather than in polyploidy.

Previous investigations (Hyppio, 1954) have suggested that the "scatter" effect of colchicine is due to a reaction taking place some time in preprophase or very early prophase. The present investigations are unable to uphold this conclusion. Rather it would appear that the scatter configuration is due to a "dilution" effect, either in doses of low concentration or in a so-called "recovery dilution effect." This view is substantiated by other investigations (Epstein, 1955). In the present investigation it was noted that scatter configurations began to appear first in time with all the doses used. In the early stages of the treatment the concentration level inside the cell is only high enough to partially destroy the spindle, thus producing scatters. As the level of concentration increases, the scatters begin to disappear, to be replaced by the clumping

effect, or full spindle destruction, provided, of course, that the original concentration in the treatment chamber was high enough in the first place to produce clumps. This must be stipulated since it was found that at the 30 ppm concentration, only scatters were achieved regardless of how long the experiment was run (Table 2). Further evidence against the preprophase effect of colchicine is contained in the results obtained with high doses. In doses of sufficiently high concentrations (75, 100 ppm and above), with the exception of the first 30 to 60 minutes following initiation of treatment, nothing but clumping of chromosomes is produced with resulting polyploidy on recovery. If scatters were a result of colchicine effect in preprophase or very early prophase, one would expect to find scatters throughout the whole experiment in substantial numbers, regardless of the concentration. Text figs. 1 and 2 show that for even the 40 and 50 ppm doses, scatters are few and far between by the time the index of effect has reached a value of 1.8 and above. An investigation using colchicine on the living stamen hairs of Tradescantia (Epstein, 1955) has also shown that the low doses cause a scattering effect, while the high doses produce complete spindle destruction and consequent clumps. It appears that the recovery c-metaphases and c-mitotic effects observed by the many previous workers in the field have been due to a "recovery dilution" effect following short time treatment with a

variety of concentrations. Full spindle destruction with its consequent clump configurations is, therefore, only demonstrable after the test material has been in sufficiently high concentrations long enough to allow the intra-cellular concentration of the drug to reach such a level as to effect this spindle destruction. From this it must follow that the best method of producing many polyploid nuclei in the root meristematic tissue, at least in Pisum, is by administering an optimum dose. An optimum dose may be described as a dose which gives a large number of clumps rapidly and which allows recovery in a comparatively short period of time so that the total number of scatters is low.

As previously mentioned, scatters, which appear first in time, and clumps, which appear later if the dose is sufficiently high, are the two configurations produced by colchicine treatment which are measurable cytological effects. In view of the fact that only scatters appeared in the low concentrations it was assumed that scatters were a less severe expression of effect. On the basis of the weights previously assigned these configurations (see observations), a linear relationship is obtained when the index is plotted on a log of time/log of dose basis (Text fig. 6). The fact that this relationship is obtained using the above mearurements can only be interpreted as indicating that the cytological effects produced must have been a direct measure of the drug effect. Thus it would appear that the cyto-

logical measurement used here was a valid expression of cytological action. These results correspond to those of Levan (1954) in his researches on the effects of colchicine on mouse ascites tumors. Although he did not use an index of effect as used here, he achieved essentially the same relationship between time, dose and effect. It might be noted here that the threshold of effect which Levan achieved in mouse ascites tumors was some 1000 times lower than that in either Allium or in Pisum. This is not surprising since numerous other investigators (Barber and Callan, 1943; Brues and Jackson, 1937; Gaulden and Carlson, 1951) have found that it takes much lower doses of colchicine to produce effects in animals than in plants.

There seems little doubt that colchicine exhibits an inhibition reaction, but does not prevent the initiation of new mitoses. The fact that colchicine, even in comparatively high concentrations, does not prevent movement of the chromosomes to the prometaphase configuration, but does appear to prevent the formation of the spindle, would seem to indicate that the prophase part of the cycle is not under the influence of the spindle. It follows from this that the action of colchicine must be restricted to a mechanism which makes its appearance as such in the cell at a time somewhere between late prophase and metaphase. What this mechanism might be is unknown. In general terms it could be called the "spindle precursor." Wilson and Hyppio (1955)

suggest that the random clumping of chromatids following "scattered c-anaphase" indicates that part of the forces responsible for chromosome movement are inherent in the chromosomes themselves. Since this clumping does not occur until the chromosomes have become telomorphic, these inherent forces could possibly be dependent on the morphological state of the chromosome.

SUMMARY

The discussion of the colchicine section of this investigation may be summarized as follows:

1. Colchicine has an inhibitory effect which seems to result in a partial or full disruption of the spindle or its precursor.
2. The mechanism which is affected must be restricted to somewhere between late prophase and metaphase.
3. The cytological effects as measured in this investigation indicate a valid and direct expression of the drug effect.

ACTIDIONE

Hawthorne (1951) and Hawthorne and Wilson (1952) have definitely shown that actidione acts as a typical prophase poison as defined by D'Amato (1949). This investigation confirms the findings of these previous investigators . . . that on a cytological basis, actidione has an inhibitory effect. This inhibitory effect acts or is reflected in two places; at preprophase and during prophase.

In the case of actidione one has two types of criteria upon which to base a measurement of cytological effect. These are ratios and configurations. As noted in the observations, the mitotic index goes down under the influence of all the doses of actidione used in this investigation. Since the drop in the mitotic index indicates an inhibition in the onset of new mitoses, the ratio set up must measure this inhibition. The type of ratio chosen was the relationship between early prophases and a total of the other stages of mitoses. This ratio turned out to be very similar between roots of a similar sampling point. As shown in Text fig. 8 the ratio of early prophases to the total of all other mitotic phases goes down almost to 0 except in the 1 and 5 ppm doses. In the case of the other doses, once they have stabilized, the drop is steady. The times that these dose curves cross arbitrarily chosen intercepts show a straight line for a time-dose relationship when plotted as log of time versus log of dose. This

suggests that the drug is acting as an inhibitor of new prophases. Since it is obvious that after four or five hours, even at the lower doses, the number of division figures in the experimental material is almost nil, it must necessarily follow that some sort of mechanism is being affected, and that this mechanism exists during interphase at least.

The cytological effect of actidione can also be determined by the anomalous configurations which actidione produces in the test material. Unlike colchicine, actidione produces only one cytologically measurable anomaly, namely the previously mentioned actidione prophase (Pl.1, Figs. 7-9). Thus the frequency of this configuration can be used as an indication of cytological effect. These actidione prophases are actually late prophases spatially. At this point they become stalled and start to become telomorphic <u>in situ</u>. Upon examining the treated material it was obvious that the actidione prophases became more pronounced and more numerous with time, and that conversely the normal prophases became less numerous. Thus it would seem natural to use as an indication of effect, the proportion of actidione prophases to normal late prophases. This type of measurement, again produces a straight line when the intercept times for each dose curve are plotted as log of time against log of dose. This indicates a inhibition effect of some kind. It suggests that a substrate is present during mid and late

prophase which is being affected by the drug. The question arises as to whether the mechanism inhibited during interkinesis is the same as that affected during mid and late prophase.

In addition to measuring the ratio of early prophases to the total of all the remaining figures, a measurement of the ratio of early prophases to normal late prophases gives curves that are similar in shape to the above. It is found that low doses reach some value definitely lower than the control, and maintain this lower level. This indicates that "valve" 2 is not closed down all the way. Factors measured here are in part combinations of factors measured in the previous systems of analysis.

When the times at which the dose curves for this measurement cross arbitrarily chosen intercepts (Text fig.9) are plotted as log of time versus log of dose, a straight line is obtained (Text fig. 10). Since a straight line is obtained with this third type of measurement which is parallel to the lines achieved for the other two types of measurements, it can be postulated that the actidione acts on the same mechanism throughout. . .in other words a mechanism exists from interkinesis through late prophase which is affected by the drug. This postulation is further supported by the fact that all the straight lines obtained are parallel and have a common slope within statistical reliability both within all the types of measurements and

also between all the types of measurements. Thus a line with a common slope can be drawn for all three categories (Text fig. 10).

This common mechanism is unknown. However, recent work (Gaulden and Carlson, 1951; Wada, 1955) suggests that the nuclear membrane has much more to do with the mechanism of mitosis than has been previously believed. The only cell structure that exists at least through mid-prophase is the nuclear membrane or wall. If the breakdown of this membrane is of such a nature that it must occur at a certain time in order for the chromosomes to carry out their proper migration toward the nuclear equator, then an interruption of this orderly breakdown must of necessity cause disruption in the chromosome movement cycle. This is further enhanced by Carlson's work with grasshopper neuroblasts (1956) in which he purports to demonstrate that the kinetochores of the chromosomes are intimately associated with the nuclear membrane up through mid-prophase or until the membrane breaks down. Wilson says (1956): "The prophase poisons which in sublethal doses have little or no effect on the spindle or on anaphase movement, do prevent formation of the prometaphase clump and so presumably interfere with forces concerned with prophase movement. Since prophase poisons appear to be associated with extended retention of the obvious nuclear membrane, it would seem likely that normal prophase movement is dependent on the nature and orderly

change in the properties of the nuclear membrane." Thus it would appear that timing is a very important matter in chromosome movement. If one postulates that in normal mitosis, the chromosomes move down the nuclear membrane to about 1/3 the distance from the pole to the nuclear equator, and that during this movement they are closely associated with the nuclear membrane via their kinetochores, then it could follow that the breakdown in this membrane allows the chromosome to migrate through the late prophase stage into a prometaphase state, at which time the spindle mechanism takes over the job of movement. The spindle substance or its precursor may exist in the center of the prometaphase configuration. Metaphase is achieved by the spindle precursor expanding to push the chromosomes out to the periphery of the metaphase plate. Subsequent polarization with its attendent physico-chemical changes might account for chromosome migration to the poles at anaphase and telophase. With this as background, it could be postulated that actidione allows the nuclear membrane to persist longer than usual, consequently the chromosomes do not migrate to the prometaphase configuration at the proper time. If the spindle cycle is at least semi-independent, it will start to act whether or not the chromosomes are in the proper place. Once the spindle mechanism has started on its cycle it becomes too late for the chromosomes to be affected by whatever the spindle substance may do. In other words the

chromosomes must be in the right place at the right time in order for their movement to be continued from prometaphase through to telophase by the spindle. Once the spindle cycle has started, chemical and physical changes may be such that the chromosomes could not become "attached" to it even if they were in the right place. . . they can't get into the prometaphase configuration under any circumstance. Thus the actidione prophase configuration represents one or both of two possibilities: (1) that chromosomes are held out in the late prophase configuration spatially because the nuclear membrane has not broken down and holds them there physically, or (2) that they are held out because the spindle substance has advanced to such a point in its development that it presents an actual physical barrier to their getting into the prometaphase configuration.

Whatever may be the mechanism upon which actidione acts there is no doubt whatever that it is not the same as that which is acted upon by colchicine. This investigation shows that the two drugs act at entirely different times in the mitotic cycle.

Before concluding the discussion of the effects of actidione, a word must be said about the low dose effects. These remarks are principally concerned with the 1 ppm dose. This dose produced a definite 50% drop in the mitotic index, but as will be noticed in all the measurements, did not reach a near 0 point as did the other doses. Actidione

inhibition is not complete as it is for the higher doses. "Valve" (1) in Text fig. 11 is not closed all the way down. This is substantiated by the fact that the ratio of early prophases to the total of all remaining figures does not go down appreciably. Thus it is possible to get a dose which shuts down mitosis only partially, but not completely. It would appear that this might be a mechanism by which mitotic activity could be controlled without stopping it completely. This has far-reaching implications since it would be reasonable to suppose from the above that an actidione-type drug might be used as an anticarcinogen.

The 1 ppm dose curve goes down with reference to mitotic index (Table 6). The ratio of early prophases to the total of all remaining figures also goes down, but not to 0. This indicated that "valve" (1) (Text fig. 11) is partially closed. The ratio of early prophases to late prophases goes down and stabilizes at a new level. This indicates that "valve" (2) is partially closed down, but not all the way. Thus some configurations are going through the normal pathway, and some are going through the actidione pathway. It can be calculated in a general sort of way about how much the mitotic activity has been decreased by this low dose (See Appendix II). Therefore it can be concluded that sub-effective doses only partially inhibit the onset of mitosis and only partially block the normal pathway through the cycle.

SUMMARY

This section of the discussion on actidione may be summarized as follows:

1. Actidione is a prophase poison as defined by D'Amato (1949).
2. Actidione produces two measurable cytological effects; one is a configuration, the other is a change in ratio of configurations.
3. Although actidione expressed its activity as two cytologically measurable anomalies, it appears that it effects only a single mechanism which is present from interphase through at least mid prophase, and this differs from the colchicine affected mechanism.
4. The types of measurements used seem to be valid measures of the cytological expression of the drug.
5. Actidione-type drugs may have anti-carcinogenic effects in low doses.

SUMMARY

1. The Pisum test was used in an endeavor to set up cytological criteria for c-mitotic and prophase poison actions on a qualitative and quantitative basis. Colchicine was chosen as the standard type c-mitotic agent, and actidione as the standard type prophase poison.
2. Root tips of Pisum sativum were exposed to various doses of colchicine and actidione respectively as continuous treatment. Cytological observations were made at regular intervals, usually hourly. All seedlings recovered when re-wrapped in wet paper toweling after treatment.
3. The effects of colchicine as reported by numerous previous investigators were confirmed. Scattered and clumped configurations were obtained, with scatters appearing first in time. It is suggested that scatters are due to a "dilution effect."
4. By assigning values to the configurations which supposedly reflect the severity of the cytological reaction, an index of effect is achieved which changes through time. This change is proportional to the dose. If the times

at which these dose curves cross arbitrarily chosen index intercepts are plotted on a log of time versus a log of dose basis, straight lines are obtained which are parallel. This would indicate an inhibition reaction of some type.

5. The cytological characteristics which a drug or chemical must exhibit to be classed as a c-mitotic agent are listed.
6. The effects of actidione as previously reported are confirmed. Actidione depresses the mitotic index at a steady rate. It also stalls the chromosomes in the late prophase configuration spatially, but allows them to progress to the next interphase morphologically.
7. Cytological measurements were made based on:
    a. a change in the ratio of configurations with time.
    b. a configuration.
    c. a combination of the above two.
8. A linear relationship between time and dose is achieved for each type of measurement on a log/log basis. Since all lines are parallel, both within and between measurement types, a single line with a slope which is the average of all the previously obtained slopes can be determined which indicates that all three measurements must measure the same thing. Again this would appear to be an inhibition reaction of some type.

9. The cytological characteristics which a drug or chemical must have to be classed as a prophase poison are listed.
10. It is obvious that colchicine and actidione do not act upon the same mechanism. Colchicine appears to act on a mechanism which is present from late prophase to metaphase, while actidione appears to have its effect from interkinesis at least through mid-prophase, and more probably, late prophase.
11. Low doses of actidione do not completely inhibit the onset of mitosis as do the higher doses. This could have important implications in the field of anti-carcinogens.
12. The importance of timing in the movement cycle of mitosis is discussed in connection with the apparent actions of colchicine and actidione.
13. A diagrammatic representation of the colchicine and actidione pathways through the mitotic cycle is presented.

CONCLUSION

It was the prime purpose of this investigation to attempt to set up cytologically distinguishable criteria by which drugs and chemicals could be set apart as either c-mitotic agents or as prophase poisons. Two drugs, colchicine, whose effects as a classical c-mitotic agent are well known, and actidione, whose effects are less well known, but which produces easily recognizable cytological anomalies were used as the test drugs. On the basis of the results reported here, it may be concluded that a typical c-mitotic agent should possess the following cytologically distinguishable characteristics:

1. There should be no inhibition of the onset of mitosis.
2. There should be a production of cytologically distinguishable anomalies.
    a. Scatters. . .signifying partial spindle destruction.
    b. Clumps. . .signifying full spindle destruction.
3. A relationship should be present such that a steady progression of events is indicated.

4. A straight line should be achieved if the times at which the various dose curves cross arbitrarily chosen intercepts are plotted as log of time versus log of dose.
5. Polyploidy or multinucleated cells or both should be recoverable.

Likewise a typical prophase poison should fit the following criteria:

1. The onset of mitosis should be inhibited.
2. The prophase stage should be "poisoned" at some time during its progress in such a way that the chromosomes do not arrive at the prometaphase configuration.
3. A measurement of the inhibition of mitosis and of the prophase poisoning effect should show a steady progression of events on a time-dose relationship.
4. A straight line should be achieved when the times at which the various dose curves pass through arbitrarily chosen intercepts are plotted as log of time versus log of dose.
5. The only possible recoverable type other than normal mitosis might be multinucleates derived from the actidione prophases which arose during treatment, but which didn't get beyond that point when treatment ended.

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APPENDIX I

SUMMARY OF MATHEMATICAL CALCULATIONS

Colchicine:

40 and 50 PPM experiment:

Slope of probit line (Text fig. 3): 0.42

Slope of probit line (Text fig. 4): 0.32

30, 40, 50, 75 and 100 PPM experiment:

Slope of 0.2 intercept line (Text fig. 6): -2.6433

Slope of 1.0 intercept line (Text fig. 6): -2.6065

Slope of 1.5 intercept line (Text fig. 6): -2.2232

Value of t for the extremes of slope: 2.3342 for two degrees of freedom.

APPENDIX II

SUMMARY OF MATHEMATICAL CALCULATIONS

Actidione:

Early prophases/total of all remaining mitotic figures (Text Fig. 10 A)

0.2 intercept slope: -0.5103
0.3 intercept slope: -0.4922
0.5 intercept slope: -0.5318

Actidione prophases expressed as % of normal late prophases (Text fig. 10 B)

30% intercept slope: -0.5083
50% intercept slope: -0.5127

Early prophases/late prophases (expressed as a ratio) (Text fig. 10 C)

1.0 intercept slope: -0.3560
2.0 intercept slope: -0.4535
3.0 intercept slope: -0.5451

Single line expressing average slope: -0.4887

Value of t for the two extremes of slope: 1.43 for two degrees of freedom.

TO CALCULATE THE AMOUNT OF SHUT-DOWN
OF VALVE (2), TEXT FIG. 11

Configuration	Number	
	1 PPM*	Control
Early prophase	44	84
Late prophase	17	11
Post prophases	52	80

E/L** actidione prophases + post prophases should = a constant.
E/L (Control) + actidione prophases + post prophases = 0.6
E/L (1 PPM) + actidione prophases + post prophases = 0.6

E/L (Control) = 7.6
E/L (1 PPM) = 2.2

The 11 late prophases obtained in the control is 12% of the total of late prophases + post prophases.

Expected late prophases in the treated material should be 12% of 69 or 8 figures. Actually realized 17 late prophases out of 69, or a difference of 9 between expected and realized.

Therefore 9/69 = 13% shut-down of valve 2.

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* Scored at 420 minutes post-initial treatment.
** Ratio of early prophases to late prophases.

APPENDIX III

A PRELIMINARY REPORT ON A RELATED EXPERIMENT

More as a matter of curiosity than anything else, an experiment was performed using actidione and colchicine in the same test solution. The Pisum test was performed as reported in the Methods section of this report.

From the results of this present investigation, a reasonable prediction can be made as to the approximate time any sufficiently high concentration of actidione or colchicine will produce full effects cytologically. Concentrations of each drug were chosen such that it could be reasonably expected that the full actidione effect and the full colchicine effect would be reached at the same time. This formed the "base" test combination and consisted of 20 ppm actidione and 75 ppm colchicine. From this, one combination of concentrations was set up such that the actidione concentration remained at 20 ppm, but that of the colchicine dropped to 40 ppm. Another was set up such that the colchicine remained as in the "base" (75 ppm), but that of the actidione dropped to 10 ppm. These will hereinafter be referred to as 20/40, 20/75, and 10/75.

A preliminary cytological examination of the effects showed the percent aberrations as listed in Appendix table I. Appendix table II summarizes the information in

relation to some of the previously used measurement criteria. Thus it can be seen that the E/L ratio for all combinations appears to steadily progress toward 0, and for at least the first two hours the progression is somewhat more rapid for the higher concentrations of actidione (20/75 and 20/40). This is to be expected if actidione is working.

The colchicine effects were scored as percent of post-prophases. It can be noted that in the 20/40 combination, the colchicine types did not appear as prominantly as they did in the 20/75 and the 10/75. This not unexpected since the dose of colchicine in the latter two is quite high.

In addition to the usual actidione and colchicine type configurations, a new one is observed. This new type is a segregation figure similar to that described by Huskins (1948) and Allen, Wilson and Powell (1950). It is to be noted that segregations appear in significant numbers in only one combination of concentrations. . . the 20/75. Although no adequate explanation of the production of such numbers can be given at this time, it does suggest that a certain combination of the proper concentrations is necessary.

Since actidione is present in high enough concentrations in all cases, eventually there will be no division figures present for either drug to work upon, thus finally everything will go to 0.

Few conclusions can be drawn from this preliminary experiment. However, the idea that actidione (a typical prophase poison) and colchicine (a typical c-mitotic agent)

tend to produce their particular effects independently appears to be further re-enforced. In addition, a third type of effect is produced, upon which, it is hoped, further investigations will be initiated.

APPENDIX TABLE I

FREQUENCY OF CERTAIN CONFIGURATIONS AFTER SIMULTANEOUS TREATMENT WITH ACTIDIONE AND COLCHICINE

TIME IN HOURS	0	1	2	3	4	5	6
20/75	%	%	%	%	%	%	%
Configuration							
E. prophase	38.0	38.5	22.5	9.5	5.0	0	1.0
L. prophase	16.0	15.5	22.5	30.5	18.0	1.0	6.0
Act. prophase*	0	0.2	9.5	24.0	27.0	42.5	48.0
Scatter	0	21.5	25.0	17.5	17.5	23.5	17.5
Segregation	0	0	1.0	4.5	5.0	18.5	7.5
Clumps	0	1.0	7.0	6.5	16.5	13.5	16.0
Prometaphase	12.0	9.0	6.0	3.5	1.0	0	0
Metaphase	14.0	6.0	2.0	2.0	3.5	0.5	0.5
Post metaphase	20.0	8.5	4.0	1.5	6.0	0.2	3.0
Unclassified	0	0.5	0.5	1.0	0.2	0.5	0.2
20/40							
E. prophase	38.0	41.5	25.0	20.0	6.0	0.5	1.0
L. prophase	16.0	16.0	25.5	32.0	19.0	5.0	7.5
Act. prophase*	0	0.2	3.5	14.5	35.5	55.0	56.0
Scatter	0	12.5	18.5	14.0	17.0	19.5	11.5
Segregation	0	0	0	0.3	0.5	1.5	0.5
Clumps	0	0	0	0.2	1.5	4.0	1.5
Prometaphase	12.0	10.0	5.5	3.5	4.0	0.5	0.5
Metaphase	14.0	9.0	13.0	8.5	9.0	4.5	3.0
Post metaphase	20.0	10.0	10.5	7.0	8.0	8.5	18.0
Unclassified	0	0.3	0.3	0.3	0.3	0.5	0.5
10/75							
E. prophase	38.0	35.0	41.6	9.5	4.5	1.5	2.5
L. prophase	16.0	17.5	21.0	29.5	20.0	17.0	7.0
Act. prophase*	0	0	4.0	19.0	30.5	51.5	54.0
Scatter	0	14.0	13.0	15.5	18.5	6.5	9.0
Segregation	0	0.2	0	1.0	0.2	0.3	1.5
Clumps	0	1.0	3.5	17.5	20.5	8.5	15.0
Prometaphase	12.0	16.5	6.0	2.5	0.5	0.3	0
Metaphase	14.0	6.5	5.5	2.0	0	0.3	2.0
Post metaphase	20.0	9.0	5.5	4.5	4.0	1.5	4.5
Unclassified	0	0.5	1.0	0.3	0	0.5	2.5

* Actidione prophase (See observations, this report).

APPENDIX TABLE II

MEASUREMENT OF EFFECT USING SOME PREVIOUSLY ESTABLISHED CRITERIA

TIME IN HOURS	0	1	2	3	4	5	6
Concentration				RATIO			
Early/late*							
20/40	2.4	2.6	0.9	0.4	0.1	0	0
20/75	2.4	2.5	0.7	0.2	0.1	0	0
10/75	2.4	2.0	1.7	0.2	0.1	0	0
Colchicine types as % post prophases**							
				PERCENT			
20/40	0	31.0	39.0	41.0	48.0	75.0	37.0
20/75	0	48.0	73.0	78.0	78.0	82.0	91.0
10/75	0	31.0	48.0	77.0	90.0	87.0	76.0
Scatters + segregations/clumps***							
				RATIO			
20/40	-	-	-	95.4	11.6	5.3	8.0
20/75	-	21.5	3.7	3.4	1.3	3.1	1.6
10/75	-	14.0	3.7	0.9	1.0	0.8	0.7

* A measure of actidione activity
** A measure of colchicine activity
*** A measure of a third type of effect, as yet unknown